EFFECTS OF DIETARY BIOTIN ON THE PHYSIOLOGY, ANATOMY AND MECHANICS OF PONY HOOF HORN

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ABSTRACT

A feeding experiment involving match-paired Treatment and Control groups of four ponies each was designed and conducted, under Home Office Project Licence number 60/01439, to test the null hypothesis that: "Dietary supplementation with biotin at a dose level of 0.12mg/Kg BW daily has no effect on the physiology, anatomy or mechanics of pony hoof horn".

Biotin supplementation caused a significantly higher growth and growth rate of horn at the midline dead centre of the wall for Treatment compared to Control ponies ($p<0.05$ by Students t-test). The pair of two older animals in the trial had significantly lower hoof growth when compared with the rest ($p<0.05$ by ANOVA). No significant difference was found between feet for these parameters and so the left fore foot of each animal was used to provide material for anatomical and mechanical studies.

Quantitative methods for measuring anatomical features which may have a functional significance such as tubule density, absolute areas and area fractions of hoof wall components, were devised. These methods were then used to give normal values for these parameters in the Control group and compared with those for the Treatment group.

Hoof horn *stratum medium* (SM) was found to consist of four distinct zones as defined by tubule density (TD). This was new, quantitative, anatomical information. The zones were referred to as Zones 1,2, 3 and 4 and were of approximately equal dorso-palmar depth into the hoof wall. In terms of their location within the depth of SM within the hoof wall, Zones 1-4 could then be respectively referred to as:

Zone:	Approximate dorso-palmar location within depth of stratum medium:
Z1	'outer'
Z2	'outer middle'
Z3	'inner middle'
Z4	'inner'

From this it was proposed that the pony hoof wall may function as a quadri-laminar ply.

In a follow-up study, a similar four-zoned pattern of hoof wall TD was also confirmed for horses.

There was an effect of biotin supplementation on tubule density with treatment animals having a significantly higher tubule density in Zone 4 of the SM ($P<0.01$ by Mann-Whitney U Test).

There was an effect of biotin supplementation on tubule marrow sizes. Treatment animals had significantly smaller mean tubule marrow sizes compared with Control animals ($p<0.01$ by ANOVA). This distinction was found to be due to a significant difference

between Treatment and Control animals in the two younger pony pairs and the differences were found in Zone 1 of the SM only.

The area fraction ratio of tubular to intertubular horn was found to be 1:3 and not 1:1 as had been estimated by other workers in the past.

The material stiffness of pony hoof horn was calculated from beam stiffnesses given in 3-point bending at 3 different moisture contents: 'fresh', 'fully hydrated' and 'dry'. There was no treatment effect on moisture content in any of these states, nor on material stiffness in the fresh and dry states. However, biotin-treated horn was significantly stiffer ($p<0.05$ by Mann-Whitney U Test) in the fully hydrated state.

Correlations between physiological, anatomical and mechanical properties were investigated using a Pearson correlation coefficient matrix. Significant correlations were found to exist between some zonal hoof wall anatomical parameters, between hoof wall stiffness and bodyweight and hoof wall stiffness and age of pony. Stiffness of the hoof wall was also significantly correlated with tubule cortex size and tubule cortex volume fraction in Zone 2 of the hoof wall and with marrow size in Zone 4.

In another follow-up study, the effect of another oral supplement (an evening primrose oil mixture, EPOM) was assessed in Army horses. Twelve horses were paired as closely as possible according to sex, age, weight, height and colour and then one from each pair was randomly allocated to treatment or control groups. The treatment group received 30mls of oral EPOM per day for approximately five and a half months, otherwise the nutrition and management regimes were the same for all horses. No significant differences ($p >0.05$) were seen between treatment and control groups for hoof horn growth or growth rate. However, there was a significant difference ($p < 0.05$) in hoof horn growth, within the treatment group only, between weeks four and eight after the start of supplementation. This revealed that the hoof capsule is capable of different growth responses when the equid is supplemented with different nutrients.

No significant differences ($p > 0.05$) were seen between treatment and control groups for any of the lipid fractions measured for the *stratum medium* from clippings of the hoof wall in these horses. However, there were substantial differences in perioplic horn lipid analyses with significant increases ($p < 0.05$) in cholesterol esters and partial glycerides and a highly significant reduction ($p <0.001$) in free cholesterol in the treatment group compared to the controls following EPOM supplementation.

Finally, preliminary work was started on Finite Element Analysis (FEA) and modelling at the macro- and micro- levels of structural organization of the donkey hoof wall because this is a useful means of predicting the effects of a change in hoof horn material or morphological properties, on the whole hoof capsule.

DECLARATION

I hereby declare that the work presented in this thesis was carried out by myself and has not been presented to any other University, or published elsewhere, with the exception of the papers published in peer reviewed journals which have been included in the Appendices.

Where ideas or diagrams have been inspired by the work of other authors but have been adapted by me, this has been acknowledged by the use of the term 'after'. Otherwise the ideas and figures used are my own.

John D. Reilly 15 October 1999

DEDICATION

This thesis is dedicated to the memory of my Father, to my Mother and to my loving family. In particular it is dedicated to my wife and to my children. Helen has been a rock of support and both she and the boys have had to endure the testing times that research brings. For the loving support of my parents and family, and to God for giving them to me, I am truly grateful.

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FRONTISPIECE

"When you can *measure* what you are speaking about, and express it in numbers, you know something about it, but when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the stage of science, whatever the matter may be".

Lord Kelvin (circa 1858)
from Murray and Stein (1958)

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ABBREVIATIONS, SYMBOLS AND GLOSSARY OF TERMS USED IN THE THESIS

In alphabetical order

*	Significant difference at 95% confidence level ie: $p<0.05\%$
**	Significant difference at 99% confidence level ie: $p<0.01\%$
***	Significant difference at 99.9% confidence level ie: $p<0.001\%$
100% HYD	100% Hydrated
Ab PAS	Alcian Blue, Periodic Acid, Schiffs Reagent
ACC	Acetyl-CoA Carboxylase
ANOVA	Analysis of Variance
ADP	Adenosine diphosphate
Af	Area fractions
AR	Aspect Ratio
ATP	Adenosine triphosphate
BB	Bearing Border
b	Breadth of beam (Width of Hoof Wall)
BM	Basement Membrane
BW	Body weight
C	Control group or animal
CA	Central axis
CB	Coronary Band
CF	Conversion factor
Co	Cortex
CoA	Coenzyme A
CO_2	Carbon dioxide
CT	Compression Testing
CV	Coefficient of Variation
d	Depth of Hoof Wall
DLNHG	Distal Limit of New Horn Growth
DNA	Deoxyribose Nucleic Acid
DW	Dry Weight
E	Modulus of Elasticity (ie: stiffness)
E_{beam}	Beam stiffness
$E_{material}$	Material stiffness
EM	Electron Microscope
EPOM	Evening Primrose Oil Mixture
F	Force
FEA	Finite Element Analysis
FMC	Fresh Moisture Content
FMC_D	Fresh Moisture Content as a Percentage of Dry Weight
FMC_W	Fresh Moisture Content as a Percentage of Wet Weight
FW	Fresh Weight
g	Grammes
G	Ground reaction force
GPa	Giga Pascals
H&E	Haematoxylin and eosin
HWD	Hoof wall depth

I	Second Moment of Area or moment of inertia
ICM	Intercellular cementing material
IFs	Intermediate filaments
IFAPs	Intermediate Filament Associated Proteins
ITD	Initial tubule density
ITH	Intertubular horn
iu	International (dietary) unit
Kcal	Kilocalories
Kg	Kilogram
l	Sample length
L	Lamina(e)
LF	Left Fore
LH	Laminar horn
LM	Light Microscope
LUT	Look up table
Ma	Marrow
MC%	Moisture Content (Percentage by weight)
MCC	methylcratonyl CoA carboxylase
MCGs	Membrane Coating Granules
MCM	Membrane Coating Material
MCS	Membrane Coating Substance
MDC	Midline Dead Centre
mg	Milligramme
µg	Microgramme
mg/Kg	Parts per million
µg/Kg	Parts per billion
MH	Medial Heel
MJ/KgDM	Mega-joules per kilogram of dry matter
mm	Millimetres
MPa	Mega Pascals
MQ	Medial Quarter
mRNA	Messenger Ribose Nucleic Acid
N	Newtons
ng	Nanogramme
NSD	No Significant Difference ie: $p>0.05$
P_{max}	Maximum point of failure
PA	Periodic acid
PAS	Periodic acid Schiffs Reagent
PC	Pyruvate carboxylase
PCC	Propionylcoenzyme A-carboxylase
psi	Pounds per square inch
RF	Right Fore
RH	Right Hind
RH_L	Reference hair line
RNA	Ribose Nucleic Acid
RPS	Reference point for sampling
S	Span

SC	Stratum Corneum
SD	Significant Difference
sd	Standard Deviation
SE	Stratum Externum
se	Standard error
SEM	Scanning electron microscope
SG	Stratum germinativum
SI	Stratum Internum
SM	Stratum Medium
sq rt	Square root
T	Treatment
TD	Tubule density
Tu	Tubule
VLa_4	Velocity at which blood lactate concentration remains at 4m mol/l
WW	Wet Weight
Z1	Zone 1 of the SM
Z2	Zone 2 of the SM
Z3	Zone 3 of the SM
Z4	Zone 4 of the SM

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CHAPTER 1

Introduction

1.1 General Introduction

Horses are reliant on the hoof horn capsule for effective locomotion. The hoof horn capsule consists of hoof wall, sole, frog and white line. This combined structure is required to withstand the effects of the physical and chemical environment, to protect the underlying sensitive structures from mechanical damage and to transmit the forces of locomotion painlessly to and from the axial skeleton (Reilly and Kempson 1992). In achieving these functions Pollitt (1995a,b) has called the hoof horn capsule a miracle of bioengineering.

The hoof wall is that part of the capsule which is visible when the foot is placed on the ground (Sisson and Grossman 1953) and is the most important part of the capsule for load bearing (Parker 1973). The horse suffers a number of defects in this part of the capsule which are described with qualitative terms such as "poor hoof wall growth" and "soft", "thin", "brittle" or "cracked" walls. These are clinical problems which manifest themselves in horses that have poor hoof wall growth, are unable to retain shoes, have cracks on the hoof wall and do not have an appropriate bearing surface. Animals displaying these symptoms are said to have "poor hoof horn quality". When underlying sensitive tissues are involved, these defects can cause pain and suffering to the horse, and place financial demands on the owner, all of which compromise animal welfare.

Despite its obvious importance for locomotion and welfare, little research into the physiological, anatomical and mechanical properties of the equine hoof wall has been carried out. As a result, there is little information on the causes of hoof wall defects and hence no evidence on which to base recommendations for rectifying them. In the absence of a sound quantitative knowledge,hoof defects are frustrating clinical conditions to treat.

The term "hoof wall quality" is persistently used in the equine world but it lacks definition. There is a need to characterise the hoof wall by objective methods and measurement in order to identify the critical factors that may contribute to function. There are many products on the equine food stuffs market that claim to

improve "hoof horn quality" by dietary supplementation but the effects that they are claimed to have on the hoof wall lack specific and objective explanation.

Hoof horn 'development', 'structure' and 'integrity' have all been reported to be influenced by dietary changes (Comben *et al* 1984, Kempson 1987, Kempson 1990). However, effectively these were individual case reports that were experimentally uncontrolled (Slater and Hood 1997), the methods used for assessment were subjective, and no quantitative data were provided to support the claims made. Objective measurement of defined features of the hoof capsule thus becomes the challenge in this field of study (Reilly 1995), and the effect of dietary manipulation needs to be assessed using controlled studies.

Objective measurement of the physiological, anatomical and mechanical properties of hoof wall in normal horses needs to be carried out. Our understanding of the interplay between structure and function within the hoof wall needs to improve, together with our understanding of whether structure, function, or both, can be manipulated by dietary change.

The aim of this thesis is given in section 1.10, however the specific objectives of the work, in order to address the overall aim were:

1. To design a controlled feeding experiment using ponies as the test species in order to assess effects on hoof horn production of biotin supplementation.

The factors taken into account when designing this experiment have come from the review of the literature in Chapter 1.

The design of the controlled feeding experiment (i.e. the "field" experimental work) was critical to the rest of the thesis in that it supplied material for subsequent testing (the "bench" experimental work).

2. To measure the physiological parameters: growth and growth rate of wall horn during the feeding experiment and to assess the effects of a test nutrient on them. This is described in Chapter 2.

At the end of the field experiment hoof horn samples were taken from a specific site on the wall of the left fore foot of each pony for objective histological and mechanical testing. Thus the third objective was:

3. To develop methods to measure objective and relevant anatomical and mechanical parameters to establish normal values for pony hoof horn, and to test the effects of a nutrient on them. These are described in Chapters 3-5.

Interactions between measured physiological, anatomical and mechanical properties were then investigated. The results of this, together with the overall conclusions of the thesis, are given in Chapter 6. Chapter 7 gives the enumerated conclusions from the thesis and Chapter 8 gives future research directions.

The next Section in this Chapter reviews the literature in relation to the subjects of structure and function of equine hoof wall horn and nutritional influence upon it.

1.2 Gross Hoof Wall Mechanics

The hoof wall is the load bearing portion of the capsule (Parker 1973, Sack and Habel 1977, Leach 1980). The hoof is subject to both static and dynamic loading during weight-bearing and locomotion respectively (Douglas *et al* 1996). This thesis considers the wall in static load bearing only. It is essential that the hoof is capable of withstanding the forces generated by ground impact (Douglas *et al* 1996) and that it allows painless transmission of forces (Reilly and Kempson 1992, Thomason *et al* 1992) without damage to the underlying sensitive structures (Leach and Zoerb 1983). Leach (1980) and Bertram and Gosline (1986) concluded that the hoof must be capable of withstanding both high velocity impacts and the transmission of forces between the ground and the skeleton.

The relationships between dermal papillae and subsequent epidermal growth, the construction of tubular and intertubular horn by the processes of cornification and keratinisation, and the way this system is influenced by nutrition, is fundamental to the study in this thesis, because it is this architecture that (has been investigated in order to) defines the normal features of hoof horn and the influence of nutrition upon them.

Dyhre-Poulson *et al* (1994) stated that the hoof must dissipate the shock wave produced at ground contact. The wall must be sufficiently rigid to prevent excessive deformation, and it must not 'fail' or break when carrying out its function. During

locomotion the hoof strikes the ground with great force and frequency. Quddus *et al* (1978) reported that the maximal load at high speed can exceed the weight of the horse with a maximum vertical force of approximately 9000 Newtons (equivalent to 2 x bodyweight), which develops during a stance of 0.1 seconds (Geary 1975) and at a frequency of up to 120 strides per minute (Hodgson and Rose 1994). This load is transmitted to the ground via the hoof wall, which has an area of about $20cm^2$, thus the hoof wall can experience stresses of up to 5 MPa (Bertram and Gosline 1986).

Heel expansion during loading helps dissipate loading forces and this occurs because the heel horn is, in effect, younger, moister and thinner than other areas of the wall. It is therefore more elastic (Kainer 1987) and so the hoof decreases the frequency and amplitude of vibrations caused by hoof impact (Dyhre-Poulson *et al* 1994).

The pressure on the digital cushion and bars and therefore on the cartilages also aids circulation and venous return and possibly assists arterial circulation to the laminar corium. Mishra and Leach (1983a,b) suggested that the extensive venous plexuses of the hoof possibly act as hydraulic shock absorbers.

Other factors involved in weight-bearing and anti-concussive mechanisms include the yielding of the distal interphalangeal joint, the descent of the distal phalanx, the flexibility of the lateral cartilages, and potential weight-bearing by the sole and the frog (Kainer 1987).

All the assumptions made in this thesis are for a "static" and not a dynamic system of load bearing and mechanics. Potential loads during standing only are considered, and not the effects of repeated loading during walk, trot or gallop. The *stratum medium* of the hoof wall is also assumed to be the principal load bearing component of the hoof capsule in agreement with Parker (1973), Sack & Habel (1977), Leach (1980), Bertam & Gosline (1986) and Douglas *et al* (1996).

1.2.1 Hoof Wall Deformation

Newton's third law of motion states that "for every reaction there is an equal and opposite reaction". Distortion of a solid object in response to an applied load generates internal forces sufficient to counter the applied load (Gordon 1976).

During normal weight-bearing the hoof deforms in a consistent pattern (Douglas *et al* 1996) which results from a compromise between the complex force changes which occur internally within the capsule and those external compressive forces acting against the ground (Leach 1980).

Several studies dating back to the last century have attempted to evaluate hoof deformation in response to loading (Miles 1863, Forringer 1889). However, these earlier studies were restricted by the technology available at the time. Nevertheless, Lungwitz (1891) who used the closure of an electrical circuit to trigger a bell in response to movement of parts of the hoof capsule, was able to arrive at a model of hoof wall deformation which has, in the main, been substantiated by latter workers including Fischerleitner (1974), Leach (1980), Colles (1989) and Thomason *et al* (1992).

The observations of Lungwitz (1891) are shown in Figure 1. 1 and can be summarised as follows:

i) An inward movement of the anterior aspect of the hoof wall.

ii) A concurrent expansion at the heels, consistent with the view of Miles (1863).

iii) A decrease in the height of the foot at the Coronary band (CB) with sinking of the heels.

iv) A flattening of the sole.

Leach (1980) concluded that the hoof wall is likely to experience forces originating from 3 sources:-

- compressive forces from the ground;
- tensile forces around the laminae;
- forces resulting from the change in form of the wall

These forces are also shown in Figure 1. 1.

Bartel *et al* (1978) proposed a model, along the saggital plane of the distal limb, to explain force transmission within the hoof. The ground reaction force, (G), is vertically orientated and considered to act through the centre of the capsule. This force counteracts the downward force of the bodyweight through the distal phalanx.

Figure 1.1: Hoof capsule movements during natural static loading (after Lungwitz 1891)

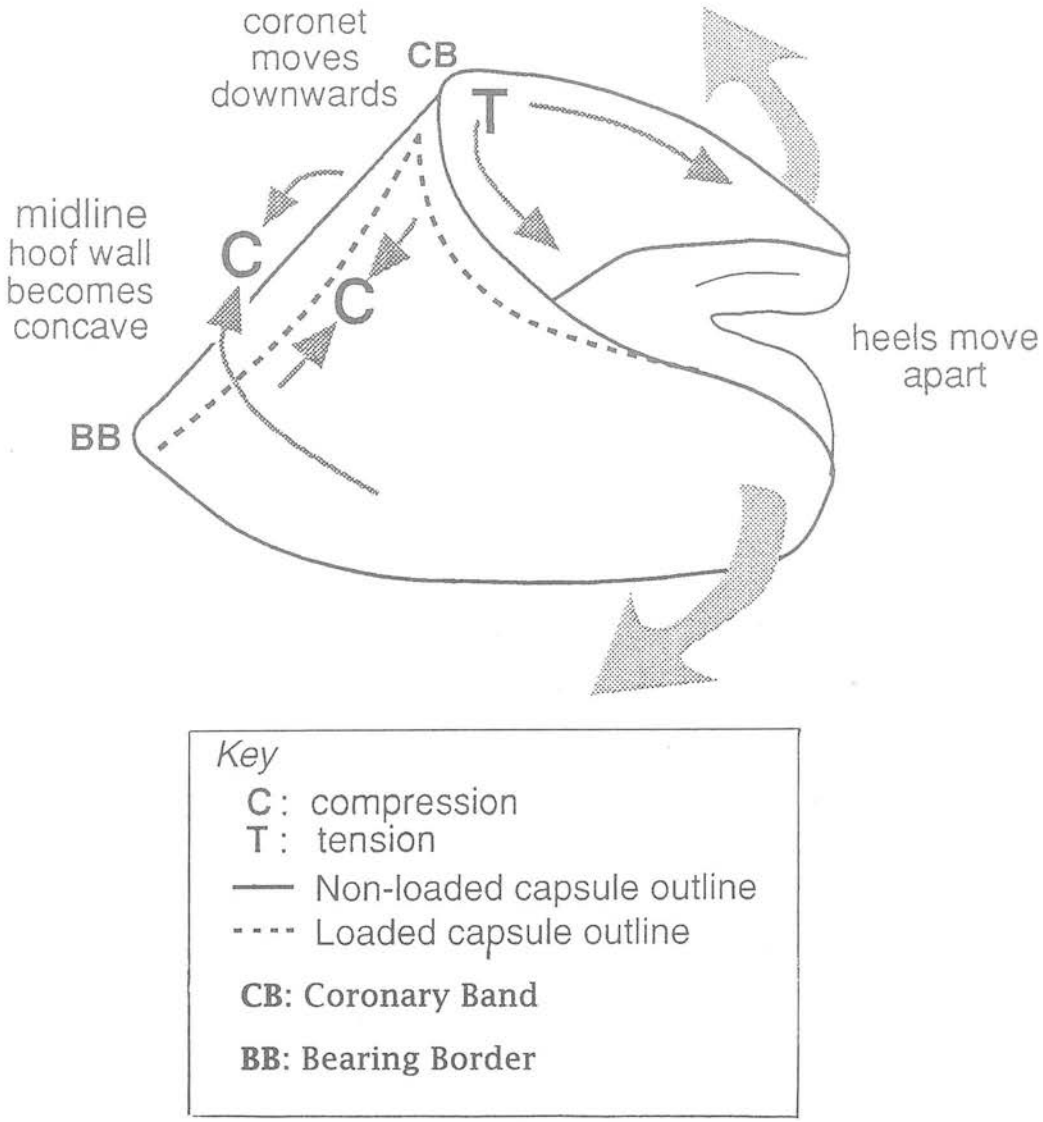
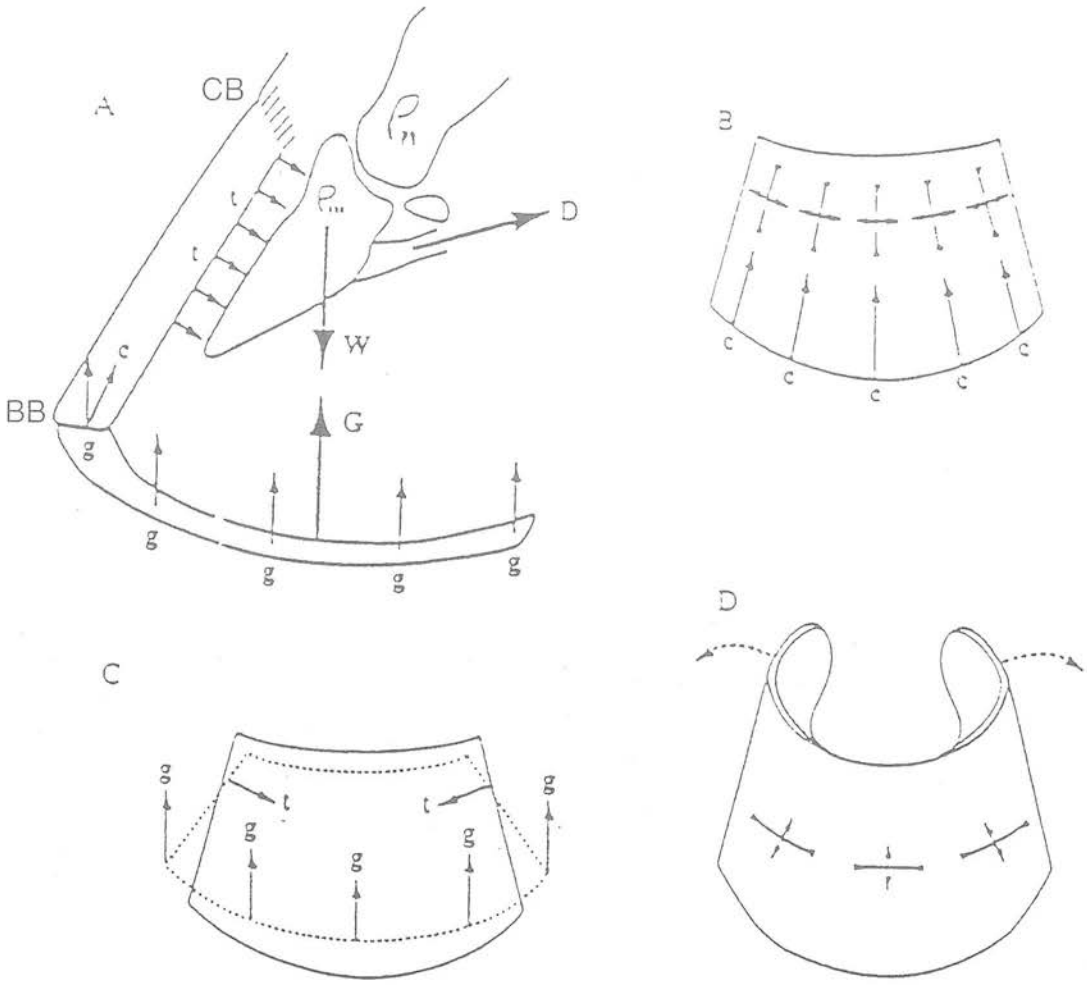


Figure 1.2: Static loading of the hoof wall
(after Thomason *et al.* 1992)



Key

A. Transmission of forces between hoof wall and skeleton in saggital section. Vectors, **g**, are components of the resultant ground reaction force,**G**, distributed around the bearing border. **t**= tensile forces.

B. Compressive forces, **c**, in the wall, in anterior view, and the strains they are likely to induce.

C. The effect of inwardly directed tensile forces, **t**, and components of ground reaction force, **g**, in anterior view.

D. Spreading of the heels and strains it might induce.

W, Downward force of the body;
D, Tensile force in the deep digital flexor tendon.
CB, Coronary band
BB, Bearing border

Components of the ground reaction force act throughout the whole of the distal bearing border of the hoof wall. As the hoof wall lies at an oblique angle to these vertically orientated components, a resolved compressive force will be directed parallel to the hoof wall and an inwardly directed tensile force, orientated in an orthogonal direction on its inner face (Thomason *et al* 1992, Douglas *et al* 1996). The former is resisted by the hoof wall whilst the latter is countered by the laminar junction. These forces are shown in Figure 1. 2.

Leach (1980) suggested that the concussive wave is probably dampened as it travels vertically through the hoof wall. This was confirmed by Dyhre-Poulson *et al* (1994) who recorded a decrease in both peak amplitude and frequency of hoof impact vibrations between the hoof wall and the first phalanx.

The magnitude of the internally-directed tensile force is thought to be increased by the action of the deep digital flexor tendon acting upon the distal phalanx. This causes a posterioventral movement of the phalanx and a synchronous displacement of the hoof wall (Fischerleitner 1974, Leach 1980, Thomason *et al* 1992). From X-ray studies, Galli (1911) demonstrated that the distal phalanx undergoes a downward rotation during weight-bearing. In his original thesis, Leach (1980) cites Mair (1973), and Fischerleitner (1974) concluding that this rotation results in a dorsoconvexity of the hoof wall. However, Fischerleitner's (1974) original illustration displays dorsoconcavity after loading. Lungwitz (1891) and Douglas *et al* (1996), conclude that a dorsoconcavity of the wall occurs in loading and the conclusion of these authors is also assumed in this thesis. Pollitt (1995a,b) confirmed this from *in vitro* photographic studies of saggital sections of the foot which revealed a concave deformation of the dorsal wall during loading. These studies confirm that the distal phalanx and the hoof wall operate as a synchronised unit (Leach 1980).

However, Leach (1980) and Thomason *et al* (1992) argued that previous models had not adequately taken account of the three dimensional nature of the hoof capsule. Compressive forces directed through the wall would result in a simultaneous tensile strain at right angles to these forces.

Expansion of the heels in response to the posterioventral movement of the distal phalanx and that of the middle phalanx results in horizontally directed

compressive forces at the toe - at right angles to those generated by the ground reaction forces. The combined effect of these forces would subject the wall to biaxial compression (Mair 1973).

Several studies have been conducted to evaluate this biomechanical model using strain gauges attached to the outer surface of the hoof in order to monitor capsule deflections during weight-bearing (Mair 1973, Colles 1989, Thomason *et al* 1992). However, there are limitations to these studies because such gauges are restricted to monitoring deflections on the outer surface of the capsule only. Information from such gauges is also site specific, yet precise information about their location is often ill defined. Thus the information given from such studies may not be representative of whole capsule dynamics.

Bartel *et al* (1978) gave a biomechanical model which indicated that the principle forces acting upon the dorsal hoof wall was compressive. However, neither Leach (1980) nor Bartel *et al* (1978) addressed the question of whether the hoof wall is subjected to compressive deformation alone, or whether bending deformation occurred or a combination of both. While strain gauge studies (Mair 1973, Colles 1989, Thomason *et al* 1992) have provided valuable information regarding hoof deformation at the surface of the capsule, they provide no information as to deformation at depth, either within or across the hoof wall.

Nickel (1938, 1939) concluded that the hoof wall was subject to compressive forces only. However, Thomason *et al* (1992) suggested that the inner aspect of the hoof capsule was likely to experience tensile forces, which suggested that bending forces may be occurring within the capsule. Rooney (1980) had earlier proposed that a complex series of bending forces occur within the hoof wall during loading. Hood *et al* (1992), using transducers capable of discriminating between bending and compressive deformation, observed that the dorsal hoof wall was subject to either pure bending, or compression and bending, during static weight-bearing. Pure compression within the wall was not recorded. This was an important finding and has influenced the choice of mechanical testing used in this thesis in Chapter 5.

Rooney (1980) proposed that the structural design of the hoof reflects the need for force resistance and energy absorption. Vogel (1989) stated that a biological

preference exists for accepting rather than preventing deformation. However, the hoof must afford protection to the underlying sensitive structures during loading. Thus the hoof capsule must possess a degree of stiffness sufficient to prevent excessive deformation whilst being resistant to capsule failure (Leach 1980, Bertram and Gosline 1986). This has fundamental implications for the type of mechanical testing that needs to be carried out on hoof material. For example, in static loading, stiffness testing is more appropriate than repeated resilience or strength testing. As a result of the findings of Hood *et al* (1992) and the general suggestions of the models in Figures 1.1 and 1.2, bending tests were undertaken for this thesis as a novel and appropriate physiological way to study hoof wall deformation. Although simplistic, this gives a first approximation of what the responses to load within the hoof capsule may be. Mechanics of the hoof wall at a micro level are considered further in Section 1.6

1.3 Anatomy of the Equine Hoof Capsule

The equine hoof is a highly evolved locomotor organ. It has a complex three dimensional structure and consists of a horny capsule made of epidermal tissue which encases bones, joints, ligaments, tendons, bursae, nerves, blood vessels, connective and fatty tissues. Nerves and blood vessels course within dermal tissue and this supplies the epidermal hoof horn capsule with its nutrients. (The hoof capsule and its contents are collectively known as the "foot". The foot comprises 2.5 bones, 11 ligaments, 2 tendons, the digital cushion (subcutis), lateral cartilages, connective tissue and the vascular and nerve supplies along with those structures directly associated with the hoof capsule (Stump 1967, Kainer 1989)).

The epidermal components of the hoof capsule that can be seen with the naked eye are the sole, frog, white line, wall and periople (Bruhnke 1931). These are shown in Figure 1. 3 and are discussed in more detail in Section 1.3.4.

The hoof capsule is a highly keratinized epidermal structure which is avascular and devoid of nerve endings (Sisson and Grossman 1953). This makes it an 'insensitive' epidermal structure (Pollitt 1990). It is composed of modified skin (Talukdar *et al* 1972) and has the same basic structure as skin elsewhere in the body, consisting of dermis, subcutis and cutis (Trautmann and Fiebiger 1957).

Figure 1.3: The visible parts of the equine hoof capsule.

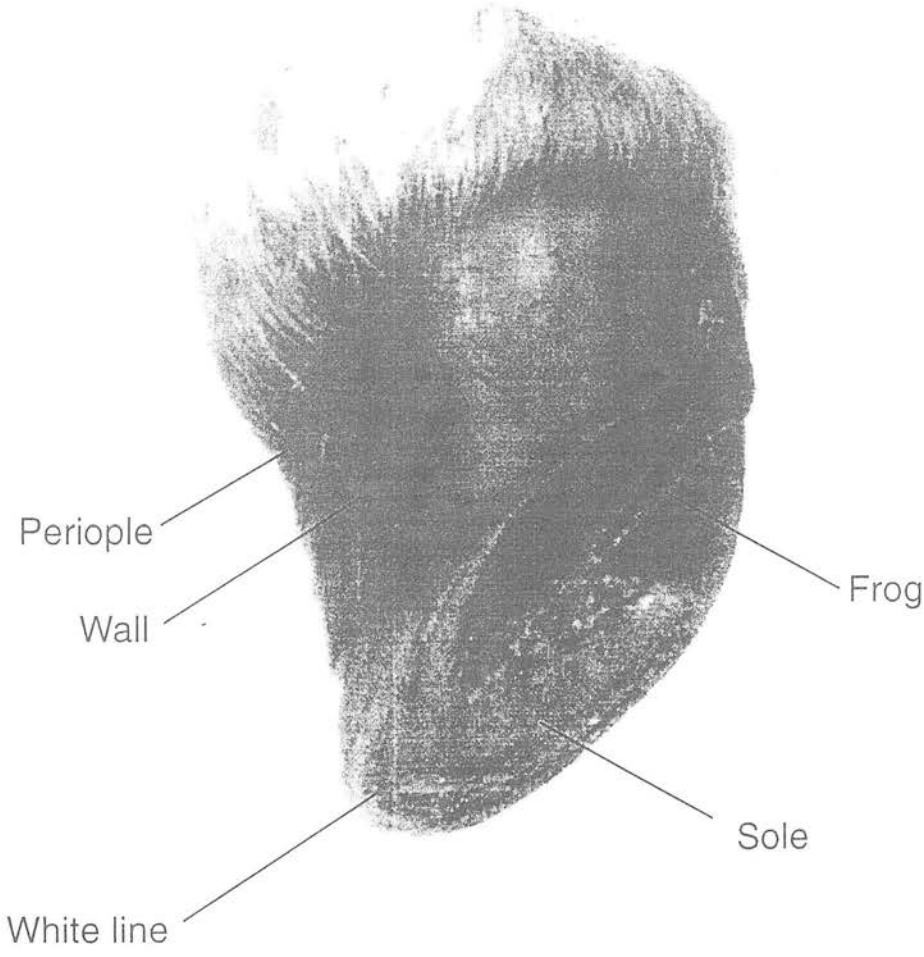
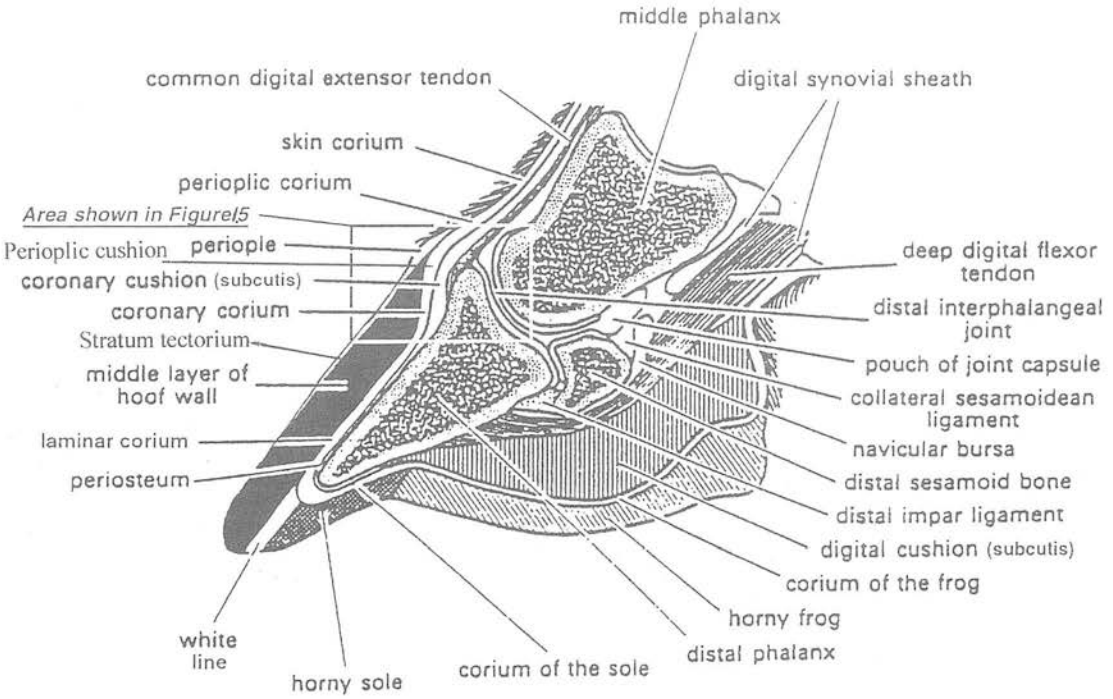


Figure 1.4: Sagittal section of the equine foot
(after Hickman and Humphrey 1987)



1.3.1. Subcutis

The subcutis i.e. digital cushion (Figure 1. 4) of the foot is of variable thickness. It is expansive at the height of the coronet and deep to the frog where it forms the perioplic and coronary cushion and the digital cushion respectively (Figure 1. 4). These cushions are composed of collagenous and elastic fibres containing fat and cartilagenous deposits. The subcutis surrounding the tendons and cartilage of the foot contains large amounts of elastic fibres (Trautmann and Fiebiger 1957). The subcutis merges into the overlying dermis:

1.3.2. Dermis

The dermis is a highly vascular tissue composed of collagenous material rich in elastic fibres. In this thesis the terms dermis and corium will be used synonymously.

The corium of the hoof forms a continuous layer and has distinct modifications according to the region in which it is located (Stump 1967). These give rise to the components of the capsule (Mettam 1896, Talukdar *et al* 1972) and thus the corium is topographically divided into five regions; the perioplic, coronary, laminar, solear and frog coria (Sack and Habel 1977, Sisson and Grossman 1953, Trautmann and Fiebiger 1957, Stump 1967) (See Figure 1. 4). The material in this thesis will principally deal with the coronary and laminar regions.

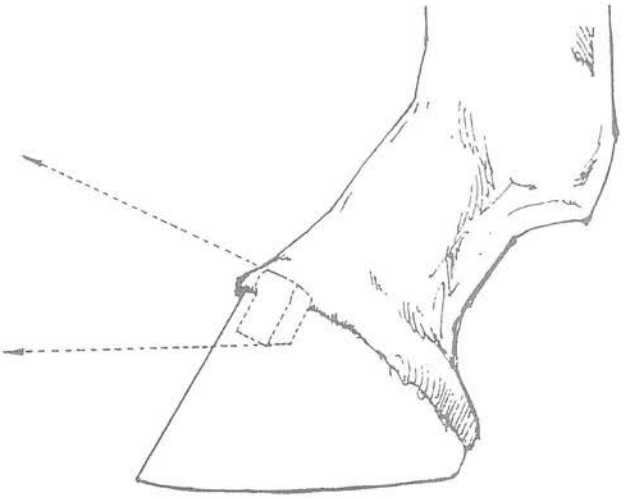
The dermis of the foot is highly modified (Dyce *et al* 1987) into either papillar or lamellar forms. The rete pegs which are characteristic of thick skin, are elongated in the foot to form dermal papillae. All parts of the corium, except for the laminar corium, are shaped into papillae and they extend in a vertical direction (See Figure 1. 5).

The dermal papillae vary both in size and shape within the dermis. They are longest within the coronary corium (Pollitt 1990) and they fit into openings or papillary sockets in the adjacent epidermal structure (Pollitt 1992) (See Figure 1. 5).

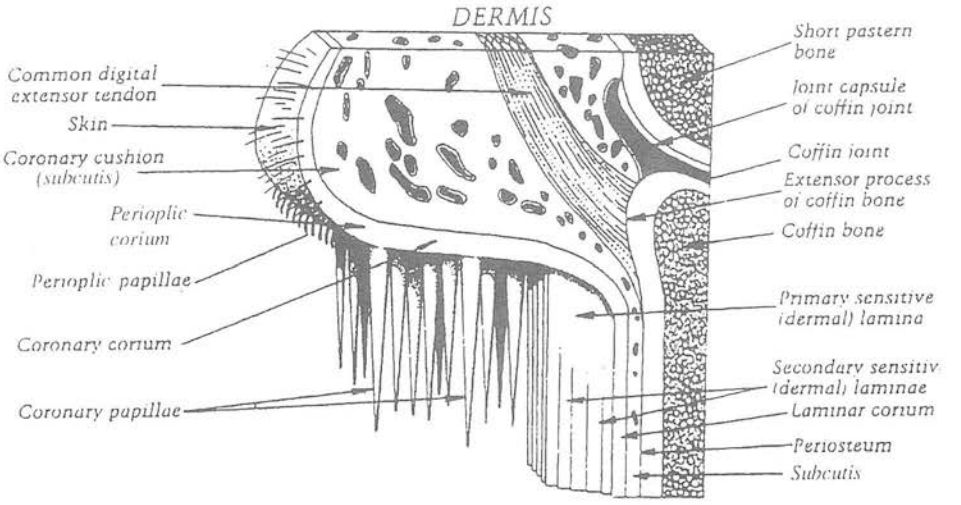
1.3.2.1. Perioplic Dermis

The perioplic dermis (see Figure 1. 4) forms a narrow band at the junction between the skin and hoof, at the height of the coronet. This spreads out caudally into the bulbar or heel corium, which in turn is continuous with the corium of the frog and sole (Sack and Habel 1977).

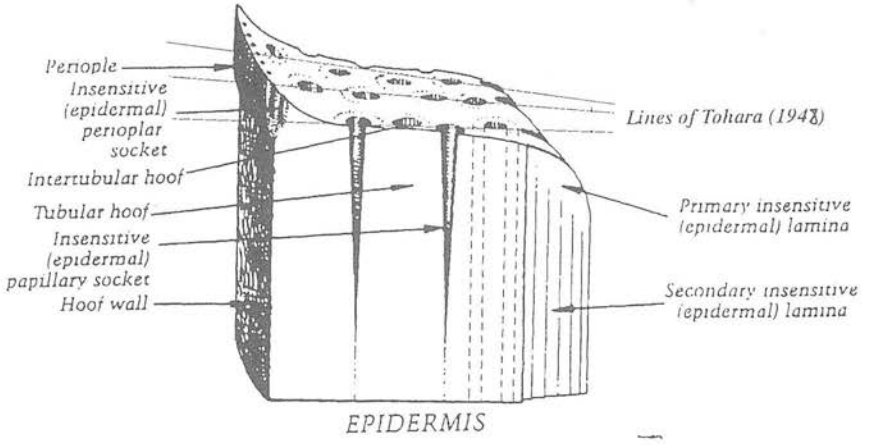
Figure 1.5: Magnified view of coronary hoof region (after Pollitt 1995)



A: Dermis: Coronary region to show epidermal papillae



B: Epidermis: Hoof wall showing epidermal sockets and lines of Tohara (1948)



The outer surface of the perioplic dermis has fine, short papillae which curve downwards into holes on the inner surface of the periople supplying it with nutrients (Sisson and Grossman 1953, cited in Stump 1967).

1.3.2.2. Coronary Dermis

The coronary corium contains dermal papillae of various sizes. Trautmann and Fiebiger (1957) observed that the papillae located over the coronary cushion were the longest and ranged from 4-6mm. The papillae became progressively more slender and shorter at 1-2mm in length at the border with the laminar corium (see Figure 1.5).

Trautmann and Fiebiger (1957) and Pollitt (1994) noted the presence of longitudinal ridges parallel to the long axis of the papillae. These were always unbranched. Pollitt (1994) suggested that these may serve to increase the surface area of attachment between the dermis and the hoof.

Trautmann and Fiebiger (1957) also noted that the papillae adjacent to the laminar corium were arranged in rows and Tohara (1948) observed that the number of horn tubule rows approximated to the number of dermal laminae at different sites around the hoof wall (see Figure 1. 5 and 1.6).

1.3.2.3. Laminar Dermis

The laminar corium is characterised by the presence of dermal laminae (see Figure 1.5). These are leaf-like structures that extend in a perpendicular plane to the papillae. There are approximately 600 primary laminae present in the horse's foot and each primary lamina has between 100-200 secondary laminae branching from it (Leach 1980, Dyce *et al* 1987). These serve to increase the surface area of attachment between the dermis and epidermis as the dermal laminae interdigitate with the adjacent epidermal laminae (Leach 1980).

The proximal end of the laminae are 'shallow' at their boundary with the coronary corium. Distally, the dermal cells at the crest of the primary laminae form accessory papillae. The distal end of each primary laminae is continued by several terminal papillae (Trautmann and Fiebiger 1957, Pollitt 1990, 1995b).

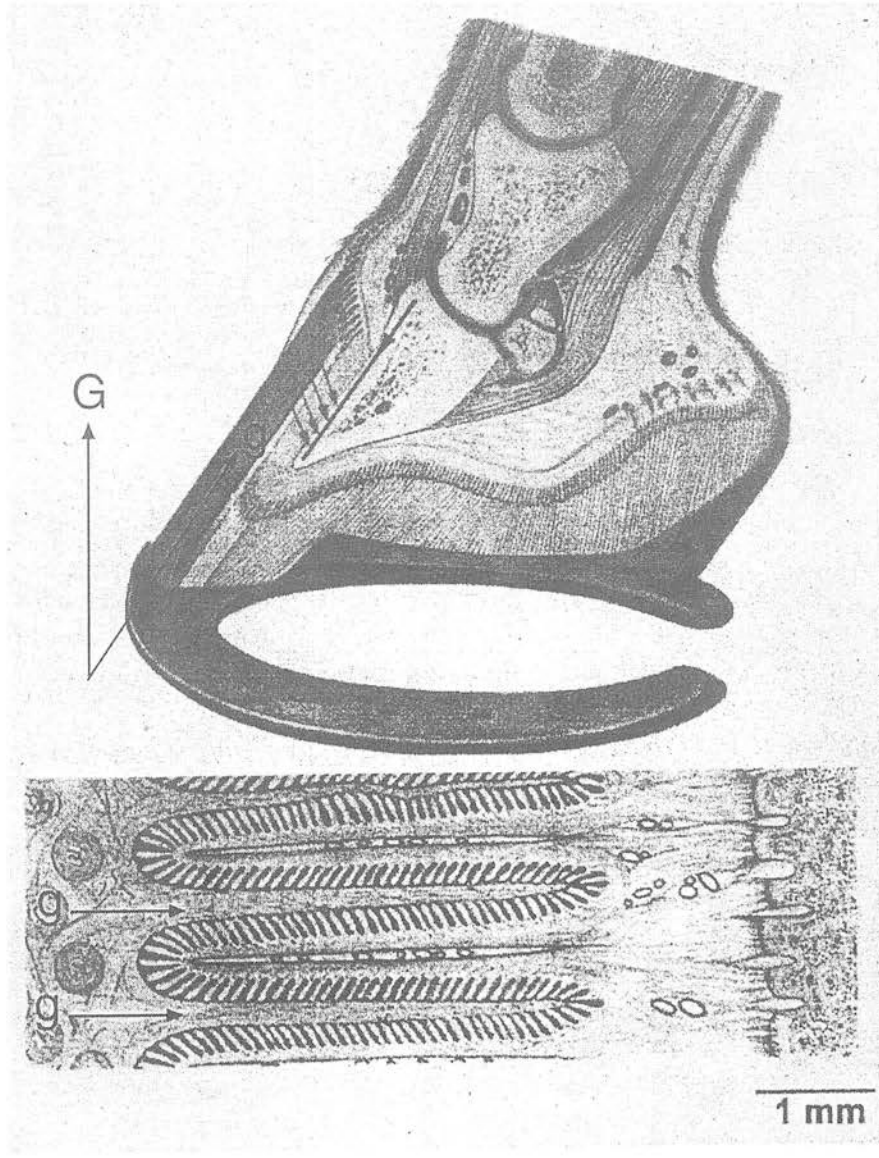
Figure1. 6: Components of the hoof wall (after Reilly et al 1996)

Description	Hoof horn capsule (seen from above)	Transverse section of wall	Cellular components (arranged as T and ITH)	Mature keratinocytes	Keratin protein
Relative magnification	x1	x10	x100	x10,000	x100,000
Structural level	Gross anatomic	Macroscopic	Microscopic	Ultrastructural	Molecular
Observation method	Naked eye	Naked eye	Light microscope	Electron microscope	X-Ray diffraction

Key

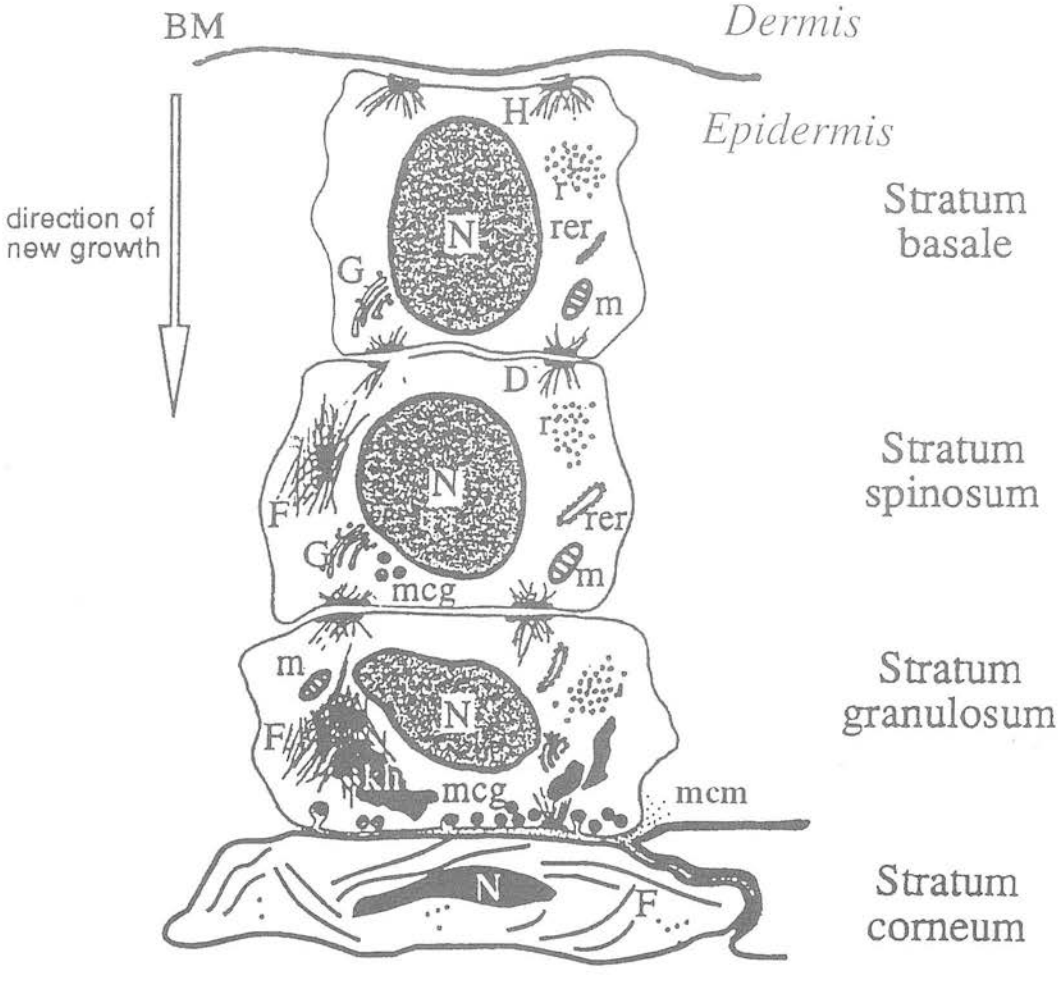
MDC	Midline Dead Centre	SM	Stratum medium
	Direction of hoof growth	SE	Stratum extumum
CB	Coronary Band	CA	Central axis
BB	Bearing Border	M	Medulla of Tubule
T	Tubular Horn	C	Cortex of tubule
L	Laminae	ITH	Inter-tubular Horn
SI	Stratum internum	y	Plane of *y* is perpendicular to planer of paper

Figure 1.7: The suspensory apparatus of the pedal bone (after Pellmann *et al* 1997)



Key
g: stress vectors within hoof wall from
G: ground reaction force

Figure 1.8: The cellular process in keratinization (after Leach 1980)



BM	Basal membrane	kh	keratohyalin
D	Desmosomes	m	mitochondria
F	Tonofilaments	mcg	membrane coating granules
G	Golgi complex	mcm	membrane coating material
H	Hemidesmosomes	r	ribosomes
N	Nucleus	rer	rough endoplasmic reticulum

1.3.3. Basal Membrane

The dermis is separated from the epidermis by a basal membrane (BM). A dense matrix of fibres connects the basal membrane of the laminar corium to the periosteal surface of the distal phalanx to form the hoof/pedal bone bond (Pollitt 1990, 1994). In this way, together with the interdigitations of dermal and epidermal laminae over a large surface area, the distal limb is suspended within the hoof, as opposed to being supported from below (Pellmann *et al* 1997). (See Figure 1.7).

The basal membrane is covered by a single layer of dividing epidermal basal cells called a germinal epithelium, known as the *stratum basale* or the *stratum germinativum*. These cells are anchored to the basal membrane by hemidesmosomes. (See Figure 1.8).

The basal cells in turn are attached to each other by desmosomes (Leach 1980) (see Section 1.4 Keratinization).

Mitotic division of this basal layer results in growth of various parts of the hoof. The subsequent process of keratinization gives rise to the *stratum corneum* or hoof capsule, which is composed of fully keratinized epidermal horn cells.

1.3.4. Components of the Epidermal Hoof Horn Capsule (See Figure 1. 3)

1.3.4.1 Sole

The sole is a concave structure occupying the space between the frog and the bearing border of the wall. The solear horn also has a tubular and intertubular form and is produced from *stratum germinativum* covering the dermal tissue arranged as papillae underlying the base of the pedal bone.

1.3.4.2. Frog

This is a readily recognised 'V' shaped structure on the underside of the hoof capsule with its narrowest part pointing forward. The palmar aspect of the frog merges into the bulbs of the heels (Dyce *et al* 1987). During footfall, some believe that the frog hits the ground first. The frog is made of a rubbery form of horn which is thought to function as an anti-concussive device, a non-slip device and possibly as an aid to blood circulation and heel expansion. In this thesis it is assumed that the frog plays no part in weight-bearing as in most equine animals it is trimmed well back. This allows the hoof wall to support the animal's body weight.

1.3.4.3. White Line

The white line is that portion of the hoof that forms the sole/wall junction distal to the dermal and epidermal laminae (Bolliger 1991, Warzecha 1993). Thus, very simplistically, the white line is the horn produced from the distal parts of the sensitive and insensitive laminae. The white line is visible on the solear surface of the hoof when it is pared (Douglas *et al* 1996). It is formed by the interdigitation of the wall lamellae with the sole epidermis (Weber 1969) and is thus the junction between wall and sole. It is composed of a pigmented and a non-pigmented component (Dyce *et al* 1987, Pollitt 1990). The former represents horn of the *stratum internum* (SI) whilst the latter is the inner most *stratum medium* (SM) (Pollitt 1990) (see Figure 1. 6).

1.3.4.4. Hoof Wall

The wall is the only part of the hoof visible in the standing horse (Sisson and Grossman 1953). The junction between the wall and the true skin is the coronary band (CB) and the border between the wall and the ground is called the bearing border (BB) (see Figure 1.1).

The wall can be divided into three regions: the toe, quarters (both lateral and medial) and the heels (Bruhnke 1931, Nickel 1938, Stump 1967). There is no precise definition of where these points are on the wall and so their position appears to be arbitrary. The wall is thickest and tallest in height at the toe and thins and shortens caudally towards the heel (Leach 1980). Pollitt (1990) observed that the medial quarter is often thinner than the lateral quarter.

A hierarchy of structure exists within the wall at the macroscopic, microscopic and ultrastructural levels (see Figure 1. 6).

The wall is said to consist of three layers: the *stratum externum*, *stratum medium* and the *stratum internum* (Bruhnke 1931, Nickel 1938) (Figure 1. 6).

1.3.4.4.1 Stratum externum (SE)

This outer layer, which is also referred to as periople proximally, is composed of horn which is a few mm thick and extends distally from the region of the CB (Nickel 1938, Leach 1980). It is composed of parallel cylinders of keratinised epidermal cells (horn tubules), surrounded by less structured intertubular horn (Dyce

et al 1987) which is also composed of epidermal keratinised cells. The periople expands caudally over the bulbs of the heels and approaches the ground surface of the foot (Pollitt 1992). The presence of keratohyaline in the stratum granulosum of the periople gives it the property of elasticity in addition to its existing properties of toughness and impermeability (Pollitt 1992). The periople scales off at a variable distance down the wall to leave a thin layer of flat horn cells, the *stratum tectorium* (Sack and Habel 1977, Dyce *et al* 1987, Pollitt and Molyneux 1990). The periople and the *stratum tectorium* are believed to have a relatively high lipid content and to play a role in controlling hydration levels within the hoof capsule (Leach 1980, Schummer *et al* 1981). Pollitt (1992) stated that its role was in reducing evaporative water loss from the hoof.

1.3.4.4.2. Stratum medium (SM)

The *stratum medium* constitutes the bulk of the hoof wall. It is the principal load bearing part of the wall (Nickel 1938, 1939, Parker 1973) and extends from the CB to the BB. Like the *stratum externum*, the *stratum internum* is composed of tubular and intertubular horn (Mettam 1896, Bruhnke 1931, Nickel 1938, 1939) which may or may not be pigmented. The tubules are arranged in parallel and run distally and continuously from the CB to the BB. (Bertram and Gosline 1987, Pollitt 1995b). The tubules are produced from the cells overlying dermal papillae and the intertubular horn is produced from cells lying in the interpapillary regions as discussed in Section 1.5 (see Figures 1.5, 1.6, 1.9 and 1.10). However, in contrast to the *stratum externum*, the coronary horn is relatively thick, up to 1.5 cm wide and "hard".

A zonal variation in tubule morphology across the *stratum medium* has been subjectively described by Nickel (1938, 1938) and Wilkens (1955, 1964). The larger tubules of the inner *stratum medium* have been described as constituting a different zone and are also referred to as the "water line" by Emery *et al* (1977). Bolliger (1991) refers to "outer", "inner" and "middle" zones of the *stratum medium*. The definition of where these zones begin and end appears to be arbitrary. Zonation of the SM is discussed in more detail in Section 1.5.

Figure 1.9: Tubular and intertubular horn formation (after Bolliger 1992)

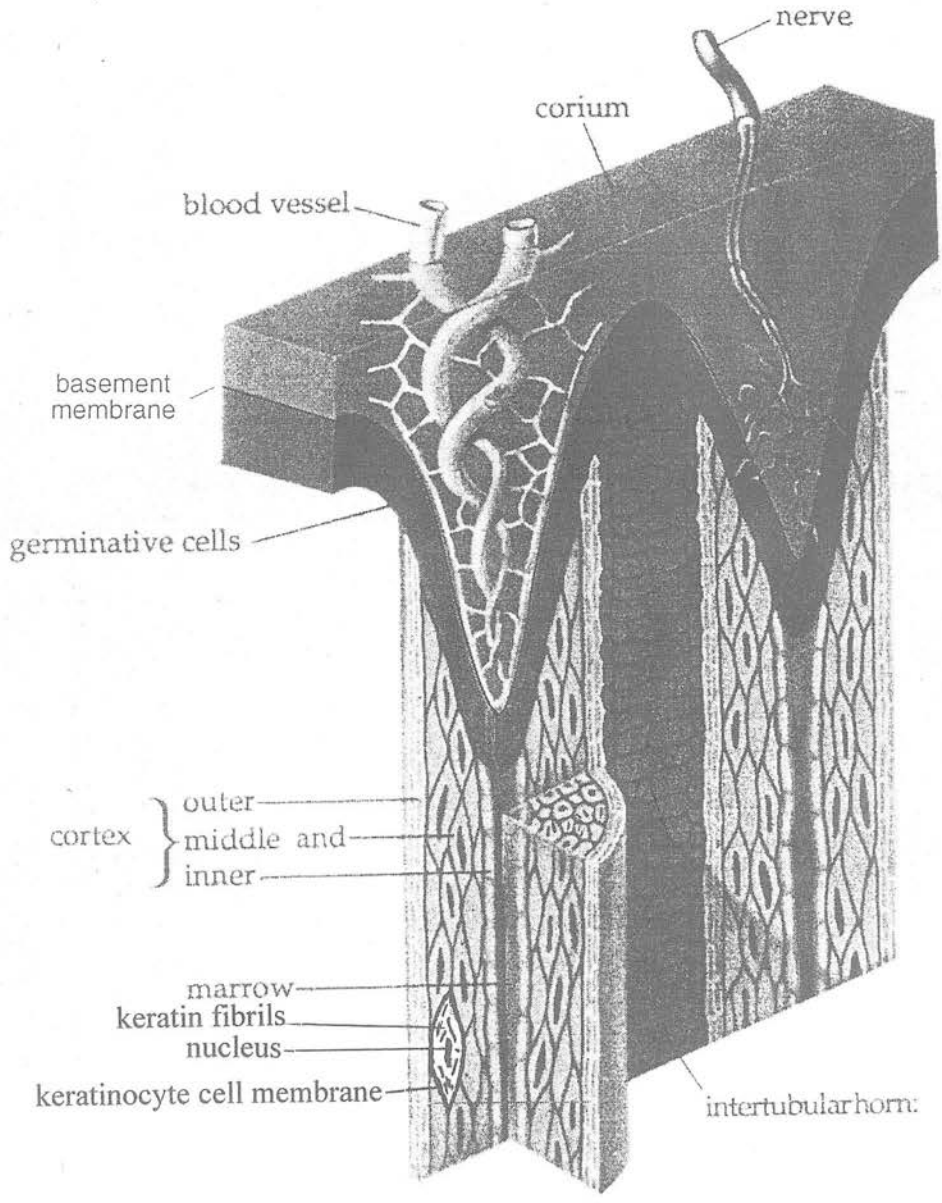
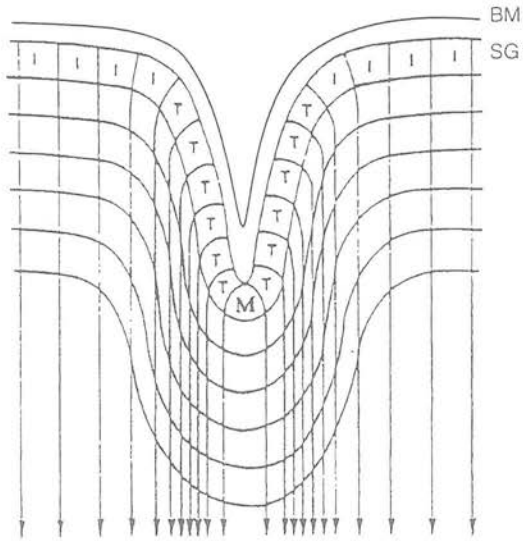
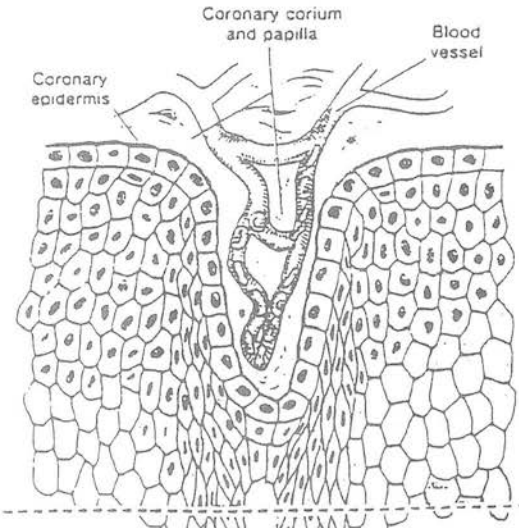


Figure 1.10: Tubular and intertubular horn formation (after Banks 1980)

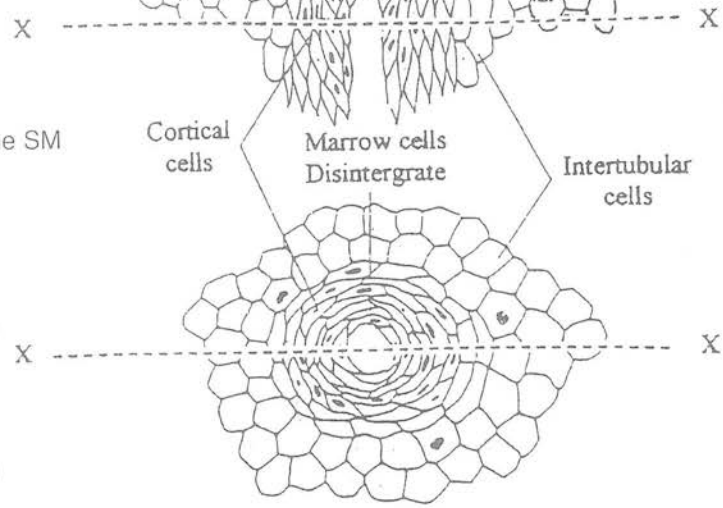
A) Schema of L-S through coronary region.
SG produces horn as marked:
I: intertubular horn
T: tubular horn
M: marrow



B) Schema to show cellular detail



C) T-S at x---x in B above to show T and IT horn of the SM



Key
L-S = logitudinal section
T-S = transverse section
SG = stratum granulosum
T = tubular horn
IT = intertubular horn
SM = stratum medium
BM = basement membrane

1.3.4.4.3. Stratum internum (SI)

This comprises the inner layer of the hoof wall and is always non-pigmented. It is composed of dermal lamellar structures which interdigitate with their epidermal counterparts to form a complex dermo-epidermal association (Leach 1980). This arrangement allows a large surface area of contact to give firm bonding between the hoof wall and, ultimately, the pedal bone via the dermal connective tissue. As a result Pellmann *et al* (1997) refer to this system as the "suspensory apparatus of the pedal bone".

This description emphasises the role of the hoof wall in weight-bearing as this structural association ultimately suspends the axial skeleton within the hoof capsule (Eustace 1990) and is thought to help the transfer of forces to and from the axial skeleton (see Figure 1. 7). However, the role that the rest of the hoof wall may play in conveying these forces to and from the *stratum internum* has been given minimal consideration in the literature.

1.4 Keratinization

The term keratinization is given to the process by which epidermal tissues are rendered tough and insoluble (Mercer 1961). It is incorrect to refer to the whole hoof wall as 'keratin' as some authors do (eg: Vincent 1990, Bertram and Gosline 1986, 1987, and Kasapi and Gosline 1997). The structural heirarchy of the hoof has been described and is represented diagrammatically in Figure 1. 6. The term keratin should be reserved for describing the fibrillar intracellular proteins only. The hoof wall can thus be seen, not to be an amorphous slab of 'keratin', but a cellular structure made up of keratinocytes containing fibrils within an intracellular matrix and interconnected by Membrane Coating Material MCM, which is believed to consist largely of lipid.

1.4.1 Cellular aspects of keratinization

The process of cellular hoof wall formation is shown as a 4 stage process in Figure 1. 8 and can be summarised as :-

1. The division of cells (mitosis) in the basal layer or *stratum germinativum* (SG) which is known as the "cornification" process.

2. The synthesis, assembly and interconnection of keratin proteins which is known as the "keratinization" process.

3. The organisation of keratin proteins into intermediate filaments to give a three dimensional cytoskeletal structure (Bolliger 1991). This is characteristic of the very resistant "hard" keratin of the hoof as opposed to the "soft" keratin of the skin (Stump 1967) and

4. The synthesis and exocytosis of an intercellular cementing substance (Bragulla et al 1992, Grosenbaugh and Hood 1993) and the formation and cross-linking of cell envelope proteins (Grosenbaugh and Hood 1993).

These stages have been derived from a succession of ideas which have evolved from the early literature based on hair, wool and skin (eg Mercer 1961). The same stages are largely assumed for the hoof since Mercer (1961) has shown that similarities exist in these processes for different epidermal structures. It is therefore reasonable to assume a model for general 'keratinous structures' that can be applied to the hoof.

The cellular architecture of the hoof is largely comprised of intracellular deposits of the structural proteins known as keratins. These are polypeptides manufactured by recognised protein-manufacturing organelles, to give discrete intracellular fibrils. They are characterised by their high cysteine content (Fraser and MacRae 1980). Nutrients are supplied to the dividing cells via the bloodstream carried within the dermis. (See Section 1.8.).

The narrow germinative region or *stratum germinativum* at the proximal border of the hoof wall consists of a single layer of live, non-keratinised cylindrical epithelial cells. These basal cells from the *stratum basale* are located at the dermo-epidermal junction (Pollitt 1992). Intercellular contact is maintained through desmosomes while basal cells are anchored to the basal laminae via hemidesmosomes (Leach 1980). The basement membrane (BM) separates the *stratum basale* from the underlying corium.

The cornification and keratinization processes give rise to histologically distinct layers. In those regions of the hoof characterized by the presence of the "hard" horn, three such layers occur. These are the *stratum basale, the stratum spinosum and the stratum corneum* (see Figure 1. 8). However, in those regions of

the hoof containing 'soft' horn an additional layer is present, the *stratum granulosum,* which exists between the *stratum spinosum* and *stratum corneum* (Ekfalck 1990, Bolliger 1991) (see Figure 1.8).

Mitotic division of the SG produces a population of polyhedral spiny cells, the *stratum spinosum*. These cells are pushed away in the direction of growth shown in Figure 1. 8 and away from the BM, (Leach 1980), to form waves of cornifying cells. These cells are interconnected by desmosomes. However an expansive 'electronoptically empty' intercellular space exists between the desmosomes (Bragulla *et al* 1992). It is in the *stratum spinosum* where the synthesis and assembly of keratin proteins takes place (Bragulla *et al* 1992).

Fibrous keratin filaments and the non-fibrous protein matrix are synthesised within each cell (Fraser and MacRae 1980). As the keratin filaments accumulate in the cytoplasm of mature epithelial cells, they become progressively linked to associated proteins, partly by means of disulphide bonds (Bolliger 1991). (This is discussed further in Section 1.4.3). The result is a stable composition of long thin keratin fibrils embedded in a surrounding matrix. This intracellular network is believed to be responsible for the cells structural properties (Bolliger, 1991).

The final product of keratinization are the cells of the *stratum corneum* which are fully keratinized cells or keratinocytes with shrunken nucleii. The organelles in these cells have also degenerated and the cells contain cross-linked keratin protein (Fraser and MacRae 1980) as an intracellular network which is partly bonded by disulphide bonds (Pollitt 1992). This network is thought to be embedded in an amorphous complex which is produced from the keratohyaline granules (Matoltsy 1976).

Keratinocytes die during the final stages of their differentiation (Fraser and MacRae 1980) although their skeletons form a tough protective layer (Bolliger 1991). Matoltsy (1976) believed that membrane coating granules discharge their contents which then act as an intercellular cement to bind the keratinocytes together.

The cells of the *stratum spinosum* contain numerous α keratin filament bundles and are located above the *stratum basale* (Eckert 1989). These cells develop cross links with each other and with other proteins via disulphide bonds.

Final maturation occurs as the keratinocyte dies but it remains surrounded by a cornified envelope (Eckert 1989). Thus the *stratum medium* is composed of mature α keratin and in this way, the hoof wall is a product of cell division from a single germinative layer of basal cells, located at the dermo-epidermal junction (Pollitt 1992).

It is believed that "keratin" cannot regenerate or remodel *in situ*. All material properties must therefore be anticipated through structural design at the time of tissue deposition (Bertram and Gosline 1987).

At the same time as keratin formation occurs specialised vesicles are produced in the cytoplasm. In the earlier literature these are referred to as Membrane Coating Granules or MCGs (Matoltsy and Parakkal 1965, Budras and Bragulla 1991). These move to the cell border and are secreted by exocytosis into the intercellular space in the upper layer of the *stratum spinosum* (Bragulla *et al* 1992). Two components within the MCGs of different species have been identified; a PAS positive glycoprotein (Hashimoto *et al* 1992) and a Sudan dye staining phospholipid (Landmann 1988, Grosenbaugh and Hood 1992a).

The final phase in the differentiation of the mature horn cell is marked by abrupt changes in both the form of the cell and the intercellular space. The horn cells flatten and expand and then degeneration of cellular organelles occurs which is then followed by cell death. This is accompanied by the production of the cell envelope on the cytoplasmic side of the cell wall. Cell envelope proteins are linked via glutamyl-lysine bonds to form a rigid cell wall (Grosenbaugh and Hood 1993). The attachment between the horn cells is achieved by the interdigitation of cellular processes and by the contents of the MCG or intercellular 'glue' which Bolliger (1991) referred to as membrane coating material (MCM) or membrane coating substance (MCS). These processes produce a tough protective layer of skeletal horn cells (Fraser and MacRae 1980). Elias (1981) has also suggested that MCGs are responsible for forming a permeability barrier.

Uncertainty still remains as to the origin of the MCM (Leach 1980). There is debate as to whether the intercellular material remains granular, thus warranting the name MCG, or whether it is diffuse, giving rise to other terms in use such as MCM or

MCS. Hashimoto (1971a,b) in man and Budras *et al* (1989) in horses suggested that the MCM is formed from the discharged contents from the exocytosed MCGs.

Matoltsy and Parakkal (1965) and Hashimoto (1971a,b) believed that the discharged contents of MCG act as "intercellular cement". This intercellular cementing function is believed by Budras and Bragulla (1990), Kempson (1990), Mulling *et al* (1994b) and Kempson and Logue (1993) to be a defining characteristic of 'good' and 'poor' horn. Using stereological techniques in bovine material, Leach (1996) investigated the proportional area (area fraction) which the anatomical components of the MCM and membrane contributed to the whole area of interdigitating cells in white line horn. Leach (1996) found no correlation between area fraction of these components with lameness state, or with laminitis or with good or poor horn.

1.4.2 Biochemical aspects of keratinization

Intra- and inter-cellular biochemical processes associated with the differentiation, maturation and death of the keratinocyte result in the formation of the *stratum corneum* of the hoof wall (Leach 1980, Bragulla *et al* 1992).

Keratins are a group of water insoluble fibrous proteins ranging in weight from 40-70Kd (Alberts *et al* 1989). Keratin filaments are one of a number of known filamentous proteins that are called intermediate filaments (IFs) and when organised in this way they form the internal skeleton of the keratinocyte (Sun et al 1985).

The structure of polypeptide chains in mammalian hard keratins is α helical (Fraser and MacRae 1980), with filaments about 7.0 nm in diameter (Fraser *et al* 1972). Keratin is thought to be preceded by a precursor molecule, prekeratin, which is required for its synthesis.

Assembly of the keratin filaments begins with the formation of a four-chain complex by two pairs of coiled-coil keratin molecules which are stabilized by hydrophobic interactions and disulphide bonds. The complex is the smallest stable polymer that can exist in solution (Eckert 1989). Proper alignment of the keratins within the keratinocyte depends upon their interactions with intermediate filament associated proteins (IFAPs) which have a higher sulphur-containing amino acid content than the keratins (Gillespie 1972).

The keratin filaments (protein chains) have a comparatively low cysteine content and are known as low sulphur proteins (Fraser and MacRae 1980). The filaments are embedded in a non-filamentous amorphous matrix of two families of proteins; one of which is cysteine-rich (high sulphur proteins) and the other is rich in glycine and tyrosine and are known as high tyrosine proteins (Marshall 1986, Bertram and Gosline 1987). The non-helical segments of the filament proteins are also rich in cysteine (Fraser and MacRae 1980).

The non-helical domains of the microfibrils and the cysteine-rich portions of the matrix are believed to be stabilised by disulphide bonds (Bertram and Gosline 1987). The remainder of the matrix has a much lower density of covalent cross-links and so its stabilisation is believed to depend on secondary cross linking mechanisms such as hydrogen bonding (Bertram and Gosline 1986).

Sequence studies of the α helical segments of the mammalian filament proteins by Crewther *et al* (1978) and Gough *et al* (1978) have revealed a repeating pattern of hydrophobic residues which favours the formation of coiled coil rope-like structures (Crick 1953). This is the α helical structure given for the helix in Figure 1. 6. It is believed that these chains are stabilised for the most part by hydrophobic interactions such as hydrogen bonding (Grosenbaugh and Hood 1992a).

Fraser and MacRae (1980) and Kitchener (1987) have stated that α keratin is a material dominated by hydrogen bonds although there are some covalent disulphide bonds within the matrix.

As the keratin filaments accumulate in the cytoplasm of the mature epithelial cells they become progressively cross-linked to associated proteins within the intracellular matrix, partly by means of disulphide bonds (Bolliger 1991). Thus, in the final stage of keratinisation, the cysteinyl residues are oxidised in pairs to yield disulphide cross-linkages (Fraser and MacRae 1980). The remainder of the matrix has few covalent cross-links and so its stabilisation depends on secondary cross-linking mechanisms such as hydrogen bonding (Bertram and Gosline 1987).

Keratinocytes die during the final stages of their differentiation although their skeletons persist to form a tough protective outer layer (Fraser and MacRae 1980). As the keratinocytes approach terminal differentiation they produce cell envelope

proteins (Rice and Green 1977). These align themselves along the cytoplasmic surface of the cell-membrane to form a rigid water-insoluble envelope for the mature keratinocyte (Grosenbaugh and Hood 1992 a,b, 1993).

Membranes of keratinocytes contain large amounts of cystine (Matoltsy and Matoltsy 1966) and proline and it is assumed that their stability is related to the disulphide bond formed by the cystine (Montagna and Parakkal 1974).

It is likely to be the chemistry of bonding such as the proportion of hydrogen, disulphide and/or sulphydryl groups that occur at the cellular level within the tissue that results in properties such as hardness, strength or toughness (Vincent 1990), and since the detailed work of Matoltsy (1976) far more is known about the biochemistry of the IFAPs.

A final step in the production of a fully differentiated keratinocyte is the linking of cell envelope proteins on the cytoplasmic side of the cell wall to form a ridged wall. These are rich in cysteine (Fraser and MacRae, 1980) and may contribute to an apparent cysteine enrichment of the cornified epidermal layers described by Eckfalck (1990) and Grosenbaugh and Hood (1992a,b, 1993). The control of cell envelope formation is not well understood but it has been shown that adequate levels of calcium are required for the activity of epidermal transglutaminase and that an increase in cellular cholesterol levels is essential for cell envelope formation in human skin (Schmidt *et al* 1991). Thus, processes interfering with cellular calcium or cholesterol levels may, theoretically, be deleterious to hoof wall quality (Grosenbaugh and Hood 1992 a,b, 1993).

The final stages in cornification also involves keratinocytes secreting lipids into the extracellular space. Phospholipids comprise a majority of the cornified extracellular matrix of the hoof wall (Bolliger 1991, Grosenbaugh and Hood 1992 a,b, 1993). This distinguishes the hoof wall from the soft, flexible epithelium at other sites where the lipid extracellular matrix is composed of neutral lipids (Elias 1983). Grosenbaugh and Hood (1992 a,b, 1993) suggest that since the nature of the lipid matrix seems to be correlated with the hardness of the wall, defects in lipid metabolism may be a source of hoof wall defects.

1.4.3 Structural and mechanical aspects of keratinization

Mammalian keratin filaments exhibit an α helical structure resulting from hydrogen bond interaction between the carboxyl and amino groups of each amino acid. This consolidates the α helical structure. Therefore, this structure is independent of interaction between the respective side chain units of the individual amino acids and hence is highly regular and stable (Mercer 1961).

Electron microscopy has formed the basis of many untested ideas about the keratinization process (Leach 1980, Kempson 1990). Ideas have changed from the simplicity of the oxidisation process of -SH cysteine residues producing cross linkages, to include also the more complex ideas of interactions between the filaments and the complimentary matrix (Rudall 1986).

Following keratin protein synthesis the process of keratin filament formation occurs. Alpha-helical monomers unite to form a dimer of two parallel helices arranged in a coiled coil (Alberts *et al* 1989). This structure results from the interaction of hydrophobic bonds and the formation of disulphide bridges (Grosenbaugh and Hood 1992 a,b, 1993).

These subsequently form the 4 chain complex of the IF, composed of 2 staggered antiparallel coiled coils. The staggered arrangement allows elongation of the filament (Alberts *et al* 1989). These in turn become arranged in thin bundles and invade the spinous processes (Bragulla *et al* 1992).

The IF tetramers associate in an helical array to produce long 'rope- like' keratin filament (Alberts *et al* 1989). The alignment of these keratins, within the keratinocyte, is dependent upon high sulphur-containing intermediate filament associated proteins (IFAPs) of the matrix (Grosenbaugh and Hood 1992a,b, 1993).

Specific IFAPs are also responsible for the attachment of the keratin filaments to the cytoplasmic aspect of the desmosomes (Grosenbaugh and Hood 1993, Chou *et al* 1997).

This results in the formation of electron-dense fibres, within the outer layer of the *stratum spinosum* and it is this tertiary structure that makes the IFs ideally suited to withstand mechanical deformation (Alberts *et al* 1989).

In this way α "keratin" is a widespread biological material consisting of stiff protein fibrils and a pliant protein matrix (Fraser and MacRae 1980). "Soft" keratin forming cells, such as those of the *stratum granulosum* typically exhibit a β keratin X-ray diffraction pattern which has a more relaxed spiralled pattern than the alphahelical pattern of hard keratins (Fraser *et al* 1972).

The mechanical properties of a biological tissue are a function of both its geometrical form and of the intrinsic properties of the constituent materials (Fraser and MacRae 1980).

Matoltsy (1976) postulated that the strength of keratin is most probably principally related to the disulphide bonds of the amorphous matrix protein which surround the filaments. He concluded that the flexibility and elastic recovery of the protective horny layer are due to the sulphur-poor filament protein (α keratin) and a sulphur (cysteine)-rich amorphous protein.

Interestingly, Grosenbaugh and Hood (1992a,b) found that the matrix intermediate filament associated proteins (IFAPs), extracted from outer hoof wall contain an even higher amount of cysteine than those extracted from laminar tissue. This therefore may also account for the increase in hoof wall stiffness from the interior to exterior portion, which is partially mediated by the hoof wall's hydration gradient.

All keratins are assembled and cross-linked intracellularly. No evidence has been obtained to suggest that filaments pass through the cell-membrane complex (Wilkens 1964; Fraser and MacRae 1980, Marshall 1986). Although this places a limitation on the possible length of a straight filament, it is thought that keratin filaments have an extremely high aspect ratio (i.e. length in relation to width) (Fraser and MacRae 1980). Under these conditions the properties of the material will be close to that of a material with continuous filaments (Wainwright *et al* 1976). This would allow the distribution of stresses throughout the material, provided the intracellular substance is not a limiting factor (Fraser and MacRae 1980).

For small strains it seems likely that the stress developed in the filaments will result from the resistance to stretching offered by the α helices (Feughelman 1971). At larger strains the molecular organisation of the filament is progressively disrupted (Astbury and Woods 1933, Bendit and Feughelman 1968). Thus the beginning of the yield region is generally agreed to be that point where the α helical arrangement of the microfibril breaks down (Fraser *et al* 1972). It is thought that the hydrogen bonds which stabilise the helical structure rupture and the helices start to unravel to form β sheet structures (Vincent 1990).

Hardening of the keratin is brought about by oxidation of the -SH groups which link the matrix protein together and probably also joins the matrix protein to the tubules via -SH groups on the surface.

The strength and chemical inertness of 'keratin' is thought to be related principally to -S-S- bonds of the amorphous matrix protein which surrounds the filaments and to the insoluble membrane protein that encases the horny cell (Giroud and Bulliard 1935).

As keratin filaments are long rod-shaped molecules, they are ideal for contributing tensile strength to the cytoskeleton (Amos and Amos 1991). Since certain IFs, such as keratins, have an additional attachment to cell junctions then this suggests that they add to the tensile strength of the whole tissue and not just the cytoskeleton (Amos and Amos 1991).

An important feature of the cytosol that augments the 'structure and strength' of keratinous structures is the presence of IFAPs. One of these, filaggrin, has been isolated from keratinizing epidermal cells. This is a basic protein of molecular weight 30kD that has a role in forming stable bundles of keratin filaments (tonofilaments) in epidermal cells by forming biochemical cross bridges.

Cysteine-rich proteins are another group of IFAPs that are found in fully keratinized skin appendages such as hair and hoof. They form disulphide bonds with cysteine rich terminal domains of certain keratins, which produces highly rigid and insoluble keratinized tissue (Bershadsky and Vasiliev 1988). This is a prime example of the way in which IFAPs can act as 'strengthening filaments' by binding alongside filamentous proteins and by cross-linking filaments into bundles and networks as

suggested by Amos and Amos (1991). The intermediate filaments, (IFs), once formed, are almost insoluble under physiological conditions (Bershadsky and Vasiliev 1988).

The IF networks in fully keratinized cells are transformed into hard and apparently inert structures (Bershadsky and Vasiliev 1988).

1.4.3.1 Composite structures

It is this constitution of IFs within a matrix which gives rise to keratins being called 'biphasic'. (Fraser and MacRae 1980). This arrangement also allows the hoof wall to be considered a "composite" structure at this level of organisation. However, the hoof wall can also be considered a composite in terms of its tubular and intertubular structure (Reilly *et al* 1996). (See Figure 1.6).

In this way, the strength of a structure such as a hoof can be attributed to the fact that the hoof wall at a cellular level, is a biomechanical 'composite'. That is, a material composed of stiff strong fibres known as the discontinuous phase, in a relatively compliant matrix known as the the continuous phase (Vincent 1992). At different levels of organisation the 'fibres' can be considered to be either the keratin filaments or the tubules (see Figure 1. 6). The 'matrix' is what Amos and Amos (1991) refer to as the cytosol which contains IFAPs. At the tissue level, as opposed to the cellular level the 'matrix' is the intertubular horn.

The 'fibre' takes forces from the 'matrix' through shear forces developed at the fibre-matrix interface (Vincent 1992). The combination of properties which the two elements of the composite give to the whole structure could not be achieved from possessing only one of them in isolation. In this way the hoof is advantaged by a composite structure.

The properties of a composite are determined by the 'rule of mixtures'. That is, the relative area fractions of its components and their respective moduli (Callister 1994). Thus, if the hoof is acting as a composite structure then an analysis of the relative area fractions of its components and how these may correlate with mechanical properties may be a fruitful approach in the quest to understand how the hoof wall functions.

1.5 Tubular and Intertubular Horn Formation

The earliest reports of horn existing in a tubular form date from Mettam (1896) although confusingly, the tubules were then referred to as 'filaments'. Now this term is reserved for referring to keratin filaments only.

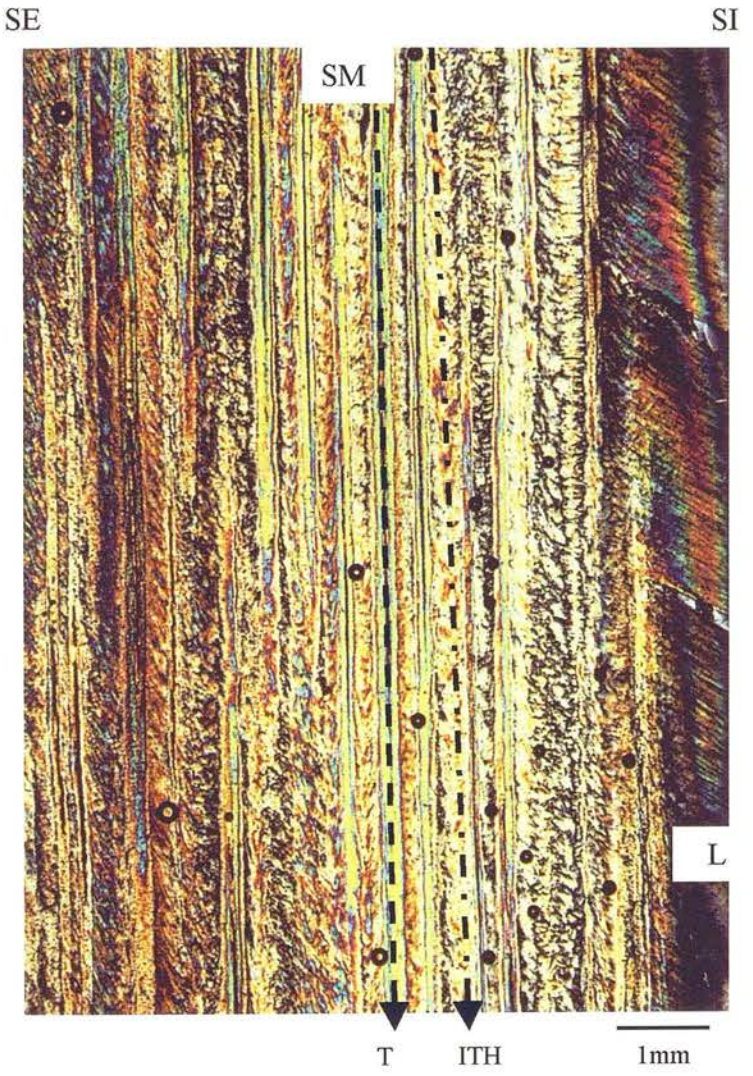
The relationship between the dermal papilla and the production of tubular horn is given in Figure 1. 5 for the level of the hoof capsular wall, and is given in more detail in Figures 1.9, 1.10 and 1.11. Keratinocytes overlying the dermal papillae produce tubular horn and keratinocytes overlying the interpapillary areas produce inter-tubular horn.

Mettam (1896) concluded that the tubular and intertubular material comprising the SM of the hoof wall was derived from the epithelial cells covering both the papillary and inter papillary areas (Figure 1. 9).

Nickel (1938 ,1939) proposed that the hoof horn of the *stratum medium* was produced by mitotic division of the basal epithelial layer, known as the *stratum basale* or *stratum germinativum*. He also concluded that the horn tubules (which he referred to as the *stratum cylindricum*) arose from the epidermal cells attached to the papillae (papillary zone) and that the intertubular horn originated from the *stratum germinativum* lying between the papillae (in the interpapillary zone).

Nickel (1938, 1939) proposed that the cells produced by mitotic division above the BM were pushed distally and proceeded to cornify (Figure 1. 8). He recognised that two distinct cell populations arose from the BM overlying the papillae: The cells of the suprapapillary epithelium, at the tips of the papillae, produced the cells of the central marrows (see Figures 1.9,1.10 & 1.11), whilst those of the papillae walls, the peripapillary epithelium, produced the tubule cortices (see Figures 1. 9,1.10 & 1.11). As these cell populations move distally, the cortical cells become arranged in cortical cords prior to the final stage of cornification, whereas the medullary cells shrink and degenerate to form a mass of loose debris in the hollow centre of the tubule, or marrow. (Nickel 1938,1939, Trautmann and Fiebiger 1957).

Figure 1.11 Longitudinal section of stratum medium to show the tubular and intertubular arrangement of pony hoof horn (10μ section polarized light – Adapted from Pollitt 1995b)



KEY:

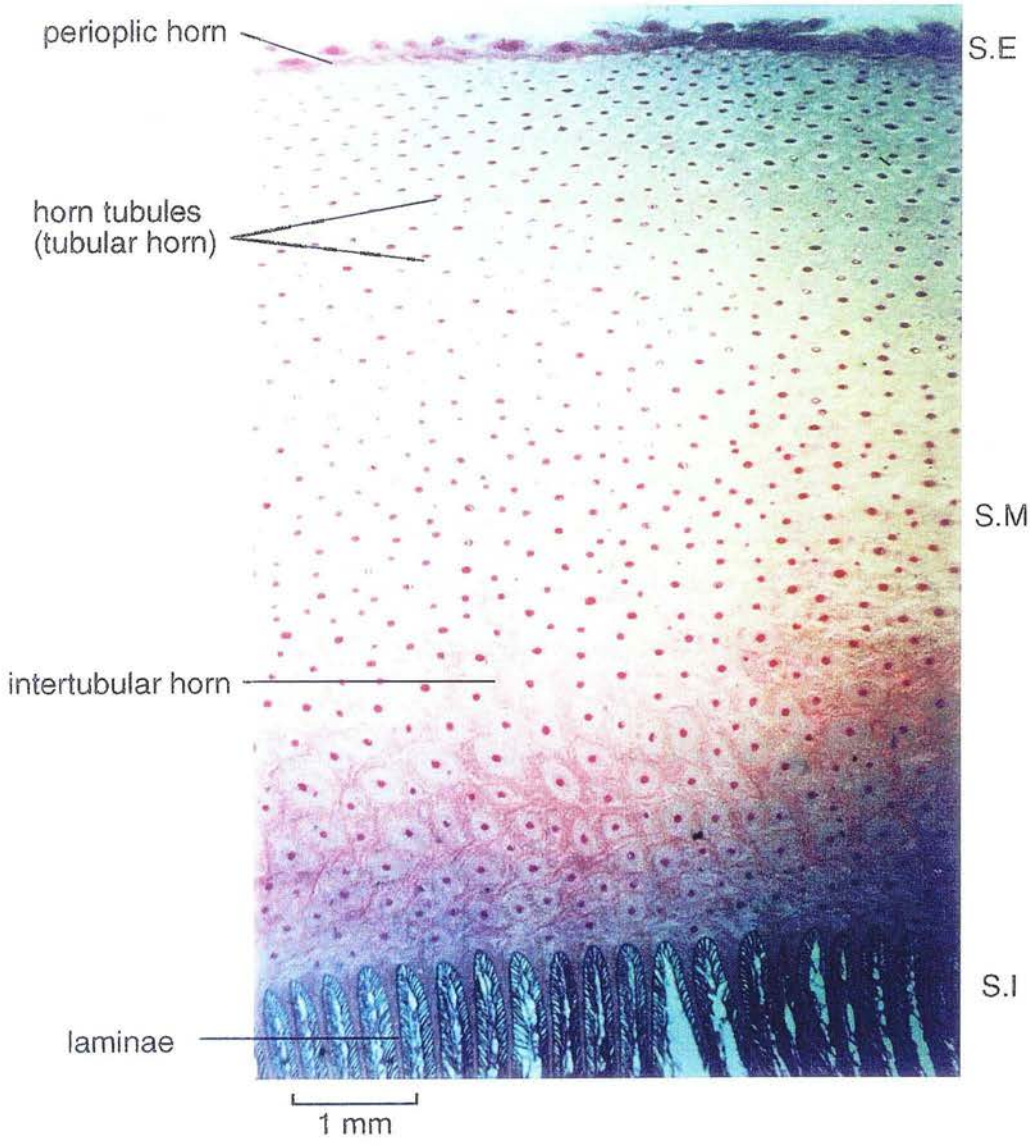
SE	Stratum Externum
SM	Stratum Medium
SI	Stratum Internum
T	Tubular Horn
ITH	Intertubular Horn
L	Lamina

Tohara (1948) proposed an alternative mode of tubule formation. On histological examination at various sites within the hoof wall, he observed that the number of tubules adjacent to the laminar corium approximated to the number of laminae. Tohara (1948) therefore suggested that tubule formation occurred by 'budding' from the free edges of the laminar corium. He believed that the tubules then migrated from the inner wall to the outer wall as a result of successive tubule formation by 'budding' from the laminae. During this migration, he proposed that cortical cell division continued within the inner third of the hoof wall, followed by subsequent cornification and shrinkage. However, the mechanisms by which tubules formed in this manner and then migrated across the wall were not precisely stated. Although the relationship between tubule rows and laminae can often be envisaged (see Figures 1.5 and 1.12) it is difficult to understand how a governing relationship between lamina and tubule could occur.

Cross sectional and longitudinal study of histological slides of coronary dermal papillae and of the laminae (Stump 1967, Leach 1980, Geyer 1980, Budras *et al* 1989, Pollitt 1990 and Bolliger 1991) have provided supportive evidence that the coronary corium is, in fact, the site of horn tubule formation as suggested by Nickel (1938,1939), Wilkens (1964) and Trautman and Fiebiger (1949). This does not support the theory of a laminar origin of tubules as proposed by Tohara (1948), and the 'sliding contact theory' (See Section 1.7.1) for distal growth of the hoof wall (Pollitt 1995b), with a contribution from keratinization of the secondary laminae (Budras *et al* 1989), is currently accepted theory.

Thus, the observations of early workers such as Nickel (1938, 1939) and Wilkens (1964) have been supported by later workers. The equine hoof wall is therefore confirmed as being composed of numerous vertically orientated tubules which run down the length of the wall, parallel to the hoof's external surface (Bertram and Gosline 1986). These are surrounded by intertubular horn (Leach 1980, Leach and Zoerb 1983, Bolliger 1991, Pollitt 1992).

Figure 1.12: Transverse section of stratum medium to show the tubular and intertubular arrangement in pony hoof horn (10μ section, Ab PAS stained)



Key
S.E: stratum externum
S.M: stratum medium
S.I: stratum internum

The SM of the hoof wall has a distinct cellular architecture made up of keratinocytes that have undergone cornification and keratinization to then be formed into a tubular and intertubular horn that has a distinct and striking form. This form is seen diagrammatically in Figures 1.6, 1.9 and 1.10 and, from histological sections in Figures 1.11 and 1.12.

There is still debate about many aspects of tubule formation such as the nature and control of the production of the marrow:

The epidermal cells at the distal end of the dermal papillae are believed to generate cells which decay and fill the marrow of the tubule (Nickel 1938, 1939, Trautmann and Fiebiger 1957, Banks 1980, Bolliger 1991). This idea is one that has perpetuated in the literature since the work of Nickel (1938, 1939) with little further study. What the final destiny of these cells is, and whether the marrow undergoes 'decay' or 'degeneration' is an area that lacks clarity.

In the interpapillary region the basal epidermal layer produces intertubular horn which links the tubules and provides a high degree of sturdiness (Klema 1937, Bolliger 1991). Once formed, the presumptive cells of the tubule cortex attach to the more fully keratinized intertubular horn cells, move distally and progressively keratinize (Leach 1980).

Leach (1980) observed that mitotic activity was confined to the proximal half of the papillae and proposed that cortical cells arising from the basal layer were added to the intertubular horn in a staggered manner. This would lead to adjacent cell cords being at different stages of cornification and keratinization during early production.

Bragulla *et al* (1994) examined bovine hoof horn formation and observed differences between mature horn cells produced from the suprapapillary epithelium compared with those produced by the peri-and inter-papillary epithelium. The suprapapillary horn cells had fewer keratin fibres, and only organelle remnants were present together with fat vacuoles and glycogen. In addition the intercellular space was increased and the suprapapillary cells produced greater quantities of membrane coating substance (MCS). The MCS in this region was also of an non-homogeneous nature which contrasted with that produced by the other horn cells. Bragulla *et al*

(1994) suggested that these differences arose due to different diffusion distances between the supporting vascular dermis and the cornifying cells.

1.5.1 Zonation of the SM based upon subjective anatomical description

The number of tubules in the equine hoof progressively increases from the few large round tubules of the inner SM (Leach and Zoerb 1983) to the numerous smaller tubules of the outer SM (Bolliger 1991). (See Figures 1.6 and 1.12). Apart from Leach (1980) who counted the absolute number of tubules in the SM, their distribution within the SM has not been quantified.

Ottaway (1955) and Bruhnke (1931), stated that the SM of the hoof wall can be divided into three regions. The 'inner' zone which is in contact with the laminae (SI), a thick 'middle' zone and an 'outer' zone which is in turn covered by a thick perioplic layer (SE). Division of the SM based on anatomical features is termed "zonation" in this thesis.

Nickel (1938, 1939), Wilkens (1964), Leach (1980) and Bolliger (1991) have all also recognised the presence of different types of tubules based upon their morphology as observed under the light microscope. They concluded that the *stratum medium* could be described as having different "zones" of varying tubule composition. All of these reports were descriptive however, with no attempts made at quantifying observed features.

Different types of tubules have been shown to be present in the horse hoof wall according to their shape and size (Wilkens 1964). The features of the cortical cells of the horn tubules allowed Bolliger (1991) to classify the tubules into three different types. Leach (1980), after Nickel (1938, 1939) also concluded that three distinct types of tubules were present within the *stratum medium* of the equine hoof wall, together with a fourth intermediate form. These are now described as if moving from 'inner' wall to 'outer' wall, that is, from SI towards SE in Figure 1. 6.

'Type 1' tubules of relatively small diameter are located between the apexes of adjacent primary laminae. These are shown again in Figure 1. 13.

'Type 2' tubules have a relatively large diameter and show distinctly cellular cortices when examined in cross section in the LM. These are shown in Figures 1.13 and 1. 14. Collectively, type 1 and 2 tubules are located in the 'inner' part of the hoof wall. 'Type 3' tubules have a variable cross section from round to oval. These are located in the 'outer' part of the hoof wall (Leach 1980), and increase in number towards the exterior (SE), becoming progressively more oval or ellipsoid in cross section in so doing. The major axes of the ellipsoids are aligned parallel to the external horizontal surface of the hoof wall (Leach 1980). These tubules are shown in Figure 1. 15.

A fourth group of tubules were observed by Leach (1980) as having an intermediate form between those of type 2 and type 3 tubules. Leach (1980) called these "intermediate tubules". These formed a narrow band within the hoof wall, separating the 'inner' and 'outer' zones. (see Figure 1. 16).

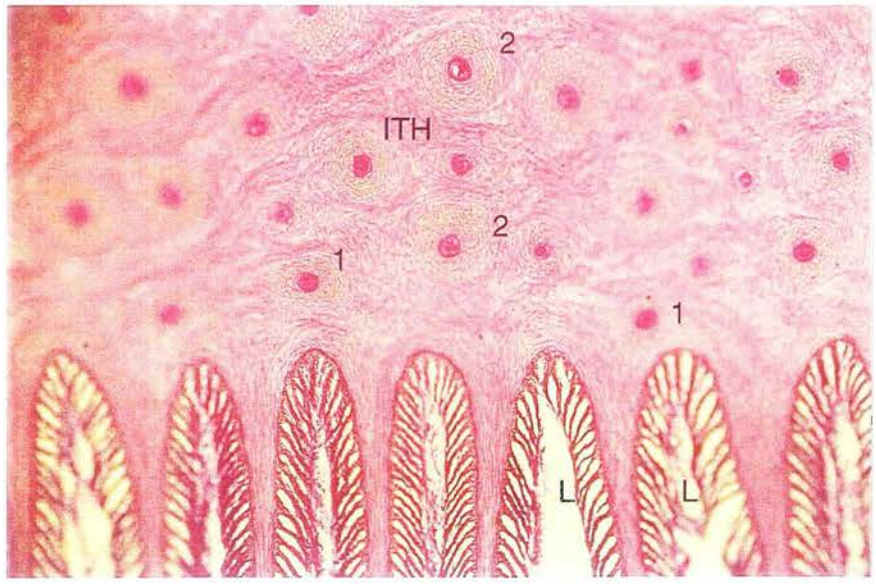
Based upon this categorisation, Leach (1980) also divided the hoof wall into 3 distinct regions:

- The inner wall/inner *stratum medium* composed of type 1 and 2 tubules and intertubular horn. This comprises the unpigmented area of the wall (Nickel (1938).
- The outer wall/outer *stratum medium*, comprised of type 3 tubules and inter tubular horn.
- The intermediate zone, representing a relatively narrow band between the outer and inner zones, containing tubules of intermediate form, together with intertubular horn.

Figure 1. 16 shows a cross section of the SM showing the tubular organisation according to Leach (1980).

Bucher (1987) studying Warmblood horses subjectively divided the hoof wall into three zones based upon eosinophilic histological staining. These zones; 'inner', 'middle' and 'outer' agree with those observed by Bruhnke (1931), Nickel (1938), Leach (1980) and Bolliger (1991). Bucher (1987), also observed that the inner zone of the SM was composed of large round tubules, whilst the middle and outer zones were comprised of oval tubules.

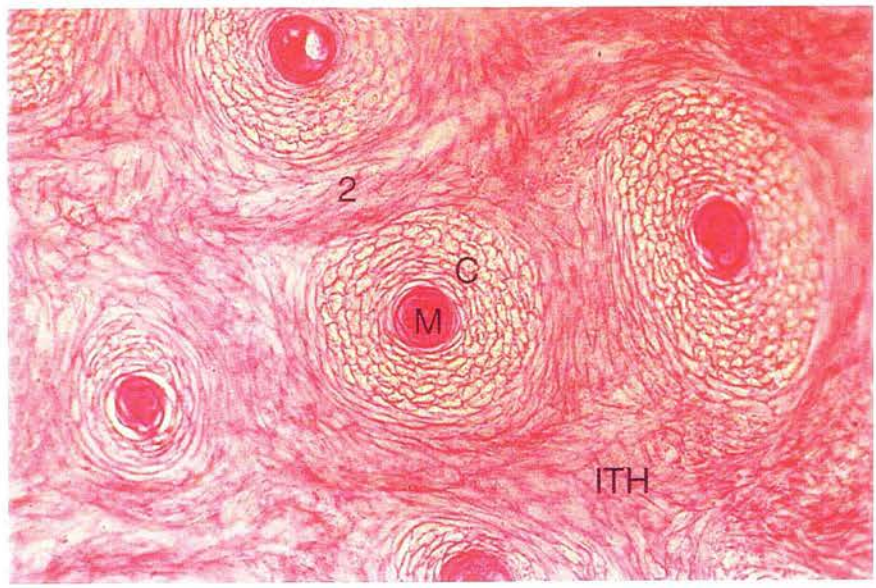
Figure 1.13: Transverse section of inner zone of stratum medium of pony hoof horn (10μ section, Ab PAS stained)



1 mm

Key
1: type 1 tubule
2: type 2 tubule
ITH: intertubular horn
L: laminae

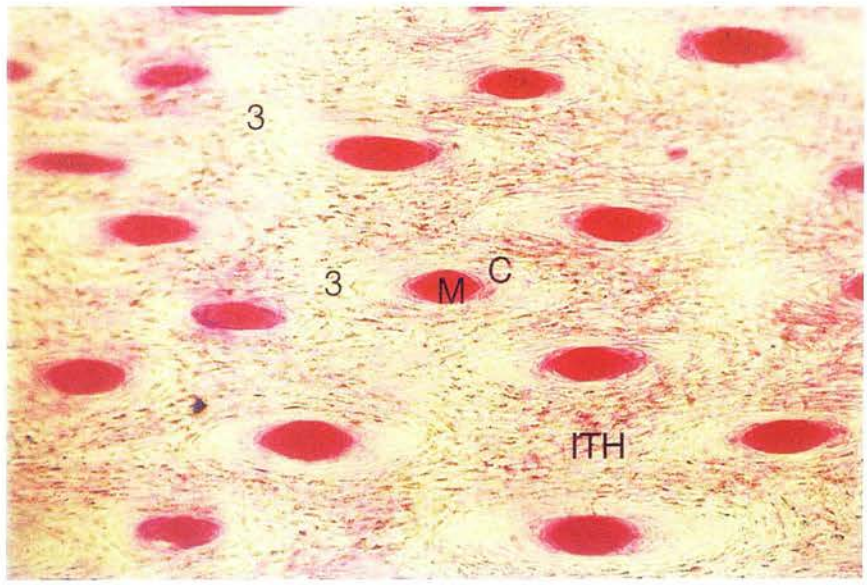
Figure 1.14: Transverse section of inner zone of stratum medium of pony hoof horn (10μ section, Ab PAS stained)



0.5 mm

Key
2: type 2 tubule
ITH: intertubular horn
M: marrow
C: cortex

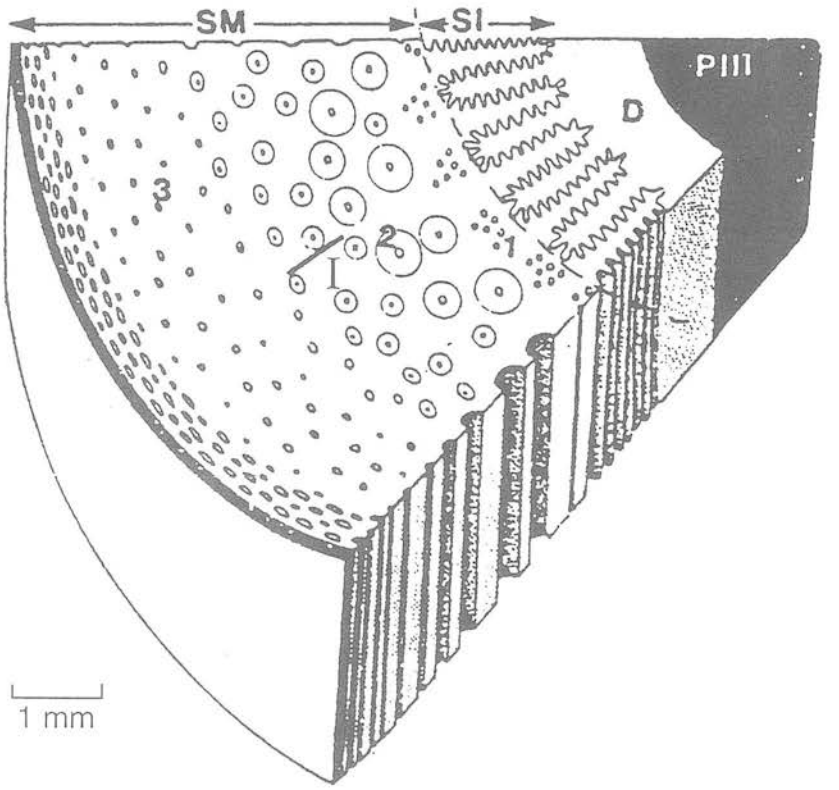
Figure 1.15: Transverse section of outer zone of stratum medium of pony hoof horn (10µ section, Ab PAS stained)



0.5 mm

Key
3: type 3 tubule
ITH: intertubular horn
M: marrow
C: cortex

Figure1.16: Transverse section of hoof wall (after Leach 1980)



Key
1,2,3: type 1,2 and 3 tubules respectively
I: intermediate zone,
D: dermis, PIII: pedal bone

Bertram and Gosline (1986) stated that the hoof wall is composed of equal proportions of tubular and intertubular horn. Although no evidence to support this was given, this idea has been perpetuated in the literature with both Thomason *et al* (1992) and Chang *et al* (1993) stating that tubules account for half of the SM.

These observations have initiated debate about the precise nature of the forces acting on the hoof wall. Ottaway (1955) cites Nickel (1938, 1939) as recognising three zones and suggests that Nickel (1938, 1939) was relating their respective thicknesses to their ability to accommodate forces. Precisely how this may be possible was not explained, but progress in the interpretation of hoof wall functional anatomy cannot be made however, until quantitative studies of features of its SM are made.

1.5.2. Objective measurement of SM features

Vincent (1990) stated that the distribution of a material within a structure is as important a consideration as the material from which a structure is composed.

Nickel (1939), Bertram and Gosline (1996), Reilly *et al* (1996) and Kasapi and Gosline (1997) have suggested that the arrangement of tubules evident within the SM of the hoof wall has functional significance and may be important in the distribution of forces within the capsule.

Changes in tubule size and shape across the dorso-palmar depth of the wall (Schummer *et al* 1981 citing Nickel 1938, 1939 and Wilkens 1964) and throughout the capsule (Nickel 1938, 1939) have been described in the horse. However, these studies have failed to quantify differences in tubule size or distribution within the SM.

Whilst the precise functional significance of zonation is unclear, it is likely that such variation confers differences in mechanical properties for different parts of the SM (Reilly *et al* 1996) which are modulated by moisture content (Bertram and Gosline 1987). This system may satisfy the different mechanical demands required across the wall (Kasapi and Gosline 1997). However, until recently these ideas had not been supported by measurement nor had the boundaries between proposed zones been accurately defined.

Kasapi and Gosline (1997) conducted a quantitative assessment of the tubules of the SM in the region of the toe. They reported the presence of two abrupt transitions in size and shape across the hoof wall at two points corresponding to a

dorso-palmar depth of 12.5 and 66% of the hoof wall depth (HWD). The latter corresponded to the intermediate zone described by Stump (1967). However, this assessment was based on only two individual samples.

Reilly *et al* (1996) have argued that the arrangement of the tubules within the hoof capsule is likely to determine hoof function, and that quantitation of the number of tubules per square mm of hoof horn, tubule density (TD), at the microscopic level is an important area of study in order to advance our current understanding of the subject.

Pellmann *et al* (1993) concluded that cortical thickness, tubule diameter and area relationships between tubular and intertubular horn determined hoof properties although no data were given to support this argument. Geyer (1980) cites Kind (1961) and Dietz and Prietz (1971) suggesting that tubular density, in association with the medullary cortical ratio, is an important parameter in determining hoof properties. Bragulla *et al* (1994) stated that the variation in tubular diameter, as well as the ratio of amount of marrow to cortex within the tubules of the SM would influence horn properties. Hofstetter (1985), summarising the work of Gunther (1974) for bovines, stated that "good quality" horn was characterised by a high tubular density, high medullary cortical ratio, and high tubular/intertubular ratio.

Rosskopf and Geyer (1987) reported horn tubule medulla enlargement in sheep with hoof problems. Similar enlargements were observed in horses reported to have poor quality horn (Geyer *et al* 1988, Geyer and Schulze 1994, Zenker *et al* 1995). Geyer *et al* (1988) reported a reduction in marrow size with biotin supplementation in one Hanovarian Warmblood horse over time, with a 50% decrease in marrow area recorded after 19 months. However, this was an observation from one horse which did not have an experimental control. Slater and Hood (1997) commented on this fact, stating that supplementation trials in the horse have suffered from a small number of trial animals and the absence of a concurrent experimental control.

1.6 Micro Mechanical Properties of the Hoof Wall

In order to carry load, and to function effectively, the hoof must have mechanisms to avoid structural failure. Thus the physical properties of the hoof wall that allow it to deform (as described in Section 1.2) will determine its functional

capabilities and limitations (Leach and Zoerb 1983, Bertram and Gosline 1987). Although a degree of 'stiffness' (that is, ability to resist bending or deformation) is required within the hoof wall to protect it from excessive deformation, the wall must not break and so stiffness must be balanced with the ability to resist fracture (Leach 1980, Bertram and Gosline 1987).

Throughout this thesis, the load taken by the hoof wall is termed 'stress' and the movement associated with taking load is termed 'strain'.

1.6.1 Stiffness, Toughness and Strength of the Hoof Wall

Resistance to deformation is termed stiffness (Bertram and Gosline 1987). Stiffness values in SI units are given the symbol of (**E**) which refers to 'modulus of elasticity' (See Chapter 5). Deformation under an imposed load is limited in composite materials because molecular interactions act to resist relative movement of the materials components (Bertram and Gosline 1987). One consequence of increasing stiffness of materials is usually a reduction of fracture resistance which causes the material to become more brittle (Bertram and Gosline 1987). Brittle materials will break because they fracture at low strains.

Resistance to fracture is described as toughness (Vincent 1990). One consequence of an increase in stiffness is a reduction in toughness which causes the material to become more brittle (Bertram and Gosline 1987).

Stiff materials resist applied forces and do not stretch or flow to any great extent. This causes any defect in the material to concentrate forces on to relatively few chemical bonds and so brings them up to failure stress, even though the overall stress within the material may be much lower than this. Consequently, rigid structural materials may fail at load levels below their theoretical ultimate strength (Gordon 1976).

The maximum stress a material can sustain before it breaks is defined as its strength (Bertram and Gosline 1986).

However, strength and fracture toughness should not be confused. They are separate entities and strength alone is not a useful parameter for describing how well a material can resist breaking (Bertram and Gosline 1986).

In addition, stiffness, toughness and strength measurements can be made under either tensile conditions, where forces are applied in diametrically opposite directions,

under compressive conditions, where the loads are applied towards each other, or under bending conditions. The conditions under which the result was generated should normally be stated. In this thesis stiffness measurements were generated for bending conditions.

1.6.2 Micro-mechanics of the Hoof Wall

The micro-mechanics of the hoof wall will be dictated in material science terms by the proportions, or area fractions, of its components. That is, by the relative proportion of component tubular or intertubular horn within a given volume of wall material. This can be taken at any level of the structural hierarchy shown in Figure 1.6. In this thesis the investigation concentrates on the tubular and intertubular form, and the possible interpretation of mechanics in this context and in terms of molecular bonding. The latter will be discussed but has not been quantitatively investigated for this thesis.

Although the hoof capsule has to function as a solid protective cover for the distal digit, it is not an absolutely rigid object (Bolliger 1991). This property is achieved in part by the arrangement of tubular and intertubular horn.

Kung (1991) reported that the mechanical properties of the hoof depend on its content of solid material. The lower the amount of 'solid' material (which Kung (1991) implies is the tubular horn as opposed to the intertubular horn), the more elastic and less rigid the hoof horn appears (Bolliger 1991). It is therefore argued that the inner wall tissue is significantly less rigid than the middle and outer wall tissue (Leach and Zoerb 1983) due to its lower amount of solid material which contains few but large round tubules (Bolliger 1991).

The keratin fibres and IFAPs form inter and intramolecular disulphide crosslinks (Fraser and MacRae 1980). Annular gap junctions aid in linking the adjacent cells to form a mechanically stable multi-directional reinforced composite (Thomason *et al* 1992).

The orientation of keratin fibres differs between the various elements of the composite and also within the tubules at different depths (Thomason *et al* 1992). If strength and stiffness is related to the area fraction of the fibre contained within the

composite, changes in the area fraction will cause changes to the mechanical properties of the hoof.

Hoof wall displays the properties of most biological materials which Fung (1968) and Wainwright *et al* (1976) describe as non-homogeneous and anisotropic. That is, they do not consistently display the same form of material throughout their structure, and their properties may be different in different directions. The organisation of the keratin without the hoof wall may be important in determining the mechanical anisotropy of the tissue (Leach 1980, Thomason *et al* 1992).

Thomason *et al* (1992) concluded that the microarchitecture of the hoof wall is an adaptation to mechanical function. Leach (1980) postulated that while the hoof wall appears to be reinforced by the tubules, the intertubular material accounts for much of the hoof wall's mechanical behaviour. This concept was supported by Thomason *et al* (1992) who suggested that the intertubular material contributes more to hoof strength, stiffness and toughness than do the tubules. Leach (1980) and Bertram and Gosline (1986) found that tensile and compressive stiffness of hoof horn are both greater in the intertubular direction, while the tubules are three times more likely to fracture.

Bertram and Gosline (1986, 1987) presented a model of hoof wall composition at the MDC in which intertubular keratinocytes crossed the axis of parallel arrangement of horn tubules. In addition, they suggested that tubular and intertubular horn are present in approximately equal proportions. Bertram and Gosline (1986, 1987) argued that the material would therefore be expected to possess similar mechanical properties in both directions. Whilst Bertram and Gosline (1986) reported similar tensile strength and stiffnesses in either direction, Leach (1980) and Douglas *et al* (1996) reported significant differences. These differences may arise from differences in the cross-sectional shape of the tubules, the arrangement of the cells within the tubules or differences in the alignment and/or number of keratin fibres within the cell.

The equine hoof owes much of its mechanical strength to its composite nature, where the individual structural components are combined in such a way that the whole is stronger and more resilient than the sum of its parts (Grosenbaugh and Hood

1992a,b). Hoof keratin behaves as a multi-directional composite capable of tolerating its usual operating strains in any direction (Thomason *et al* 1992). This mechanical behaviour also allows the material to withstand any unpredictable loading situations when the hoof contacts irregular substrates.

However, the relationships between tubular, cortical, marrow and intertubular components of the hoof wall and mechanical properties have not been resolved satisfactorily, as specific studies have not been carried out that attempt to relate mechanical properties to area fraction of component.

1.6.3 Effect/Function of Tubules

Leach (1980) attempted to interpret his histological and mechanical findings in terms of a possible model of hoof wall function. Whilst this model does not give a definitive solution to modelling the function of the hoof wall, it represents a starting point from which further studies can proceed.

Leach (1980) suggested that the hoof must have the ability to absorb strain energy and deform in order to prevent fracture due to crack propagation. However, the hoof must avoid excessive deformation, and therefore the hoof wall must balance these two considerations. Leach (1980) believed that a tubular and intertubular organisation, with its stiffness modulated by water content gave the hoof this capability.

Leach (1980) established that the stiffness of the outer hoof wall under vertical loading was twice that of the inner hoof wall. This suggested that the outer wall is capable of effectively resisting compressive forces.

Leach (1980) suggested that the elliptical tubules of the outer zone have such a shape in order to reduce the effect of horizontally directed forces. Furthermore the cortex at either end of the major cross sectional axis is thickened at the point where the greatest stress would occur whilst also serving to resist the effect of horizontal compressive stress.

Nickel (1938, 1939) suggested that the tensile forces generated within the intertubular horn as the pedal bone moves during normal static loading, are translated to the tubules which would generate axial (i.e. proximo-distal) tension in the tubule that would resist the effect of axial compression upon it.

Jeronimidis (1980) gave a thesis on the tubular form in wood which may be related to the hoof wall. He argued that the tubules in wood may be prevented from buckling due to the support of the cortex of keratinised cells which surround their marrows, as in the hoof wall. A hollow tube can fail in compression by buckling over its entire length or by local buckling over part of its length in the wall. However, the support given by neighbouring cells will, in general, prevent the first from happening in wood (Jeronimidis 1980).

Kasapi and Gosline (1997) were intrigued by the tubular structure of hoof horn. They came to the conclusion that tubules only function in crack re-direction within the SM only. To follow on from this work, Kasapi and Gosline (1998) conducted experiments to test the anecdote that tubules functioned in water transport within the SM. The tubular form was not found to be associated with hydration or dehydration functions (Kasapi and Gosline 1998).

1.6.4 Moisture

Lambert (1966) stated that the governing factor in hoof health is moisture. Miyaki *et al* (1974) found no significant difference in water content between fore and hind hooves, nor between moisture contents of hooves in four year olds and those horses over six years of age. Leach (1980) and Douglas *et al* (1996) have investigated the percentage moisture content (MC%) by weight in samples of hoof horn. Values for MC% varied from 17% to 35.5% and one factor that may cause this variation is the site within the hoof wall from which the sample was taken. The *post mortem* MC% of the SM of the hoof wall at the mid-toe region from coronet to sole averaged 27.8% by weight according to Butler and Hintz (1977). MC% for the wall at the sole border was lower, at 27.1%, than at the coronary border, at 29.1%.

Within the equine hoof wall two hydration gradients exist (Bertram and Gosline 1987). Firstly, there is a horizontal gradient in which the outermost surfaces of the hoof have a lower hydration level than the adjacent interior vascularized dermis from which hydrating fluids originate. Secondly, there is a vertical gradient, in which the concentration of moisture decreases from the proximal germinative region at the coronary band to the distal contact surface at the bearing border (Bertram and Gosline 1987). There is no MC% gradient medio-laterally within the hoof wall (Leach 1980).

Leach (1980) established that stiffness was negatively correlated with water content in the outer wall, with horizontally loaded stiffness (E) value being more affected by water content than the vertically loaded E . Hence in hoof wall with a low moisture content the lateral E would be substantially higher than the vertical E. Hence the hoof demonstrates anisotropic material properties with the outer wall under normal hydration levels being stiffer in the horizontal direction.

Leach (1980) stated that the interior-exterior hydration gradient results in the inner wall possessing a low E value and therefore is likely to deform under compression more readily than the outer wall.

Wainwright *et al* (1976) stated that the rigidity of keratin is believed to be inversely related to water content perhaps by affecting the stability of the α helical microfibrils of the keratin fibre.

Camara (1970) demonstrated a positive relationship between the level of horn moisture and the degree of wear in bovine hoof horn and suggested that an increasing moisture content increased the rate of wear. Dietz and Prietz (1981) supported these findings and proposed that the lower the water content the harder the horn and the less prone it is to abrasion and lesions.

These findings may be explained by the theory proposed by Bertram and Gosline (1987) who stated that the properties of keratinous materials are strongly influenced by their hydration state. They proposed that in the absence of water extensive hydrogen bonding occurs between the polymers in the matrix, and that this secondary cross-linking decreases the mobility of the matrix. In a fully dehydrated state the matrix would become a 'rigid polymeric glass'. That is, a stiff, cross-linked, brittle material that would break at low strain. Alternatively when the material is fully hydrated, this secondary bonding is much less. This gives the matrix polymers greater freedom of movement and the ability to rearrange under load. Thus, Bertram and Gosline (1986) suggested that decreased hydration near the external surface may cause hooves to crack.

From this is can be seen that the site of hoof wall sampling will dictate its moisture content and that in order to compare material mechanical properties such as stiffness which can be modulated by moisture content (Betram and Gosline 1987), the

effects of moisture content must be experimentally controlled. Bertram and Gosline (1987) found the MC% of hoof horn at 100% hydration was 40.2% by weight, and Kasapi and Gosline (1997), who used a 97% relative humidity (RH) chamber to control moisture content in samples, found MC% of 48%, 41% and 35% for inner, middle and outer hoof wall respectively. Butler and Hintz (1977) have suggested that further research is needed to determine factors other than moisture content which affect hoof strength.

1.6.5 Pigmentation

Pigmentation has been traditionally implicated as an important factor in the durability of horse hooves (Wiseman 1973, cited in Leach and Zoerb 1983).

However, researchers have disagreed with traditional dogma on pigmentation. Weiser *et al* (1965) and Miyaki *et al* (1974) and Leach (1980) reported that there was no significant difference in the water content of pigmented and non-pigmented hooves. Although Feder (1969) stated that pigmented horn was 30% more resistant to grinding methods in the laboratory than non-pigmented horn. Dinger, Goodwin and Leffel (1973) found no significant differences in hardness between pigmented and non-pigmented hooves from the same horse. Chemical composition was studied by Weiser *et al* (1965) who also found no significant differences between pigmented and non-pigmented horn.

For these reasons thesis pigmentation is not considered an important factor in determining mechanical properties of the hoof wall in this thesis.

1.7 Growth and growth rate of the hoof wall.

The hoof wall grows throughout the life of the horse. Mettam (1896) reported that hoof wall growth occurred proximo-distally from the coronary band (CB) to the bearing border (BB).

The importance of hoof wall growth rate was recognised by Caulton Reeks (1906) who mentioned that the slow hoof growth rate found, even in the normal horse, is frustrating for the veterinarian attempting to effect any change.

At the BB the wall is lost by abrasion or wear or, if the horse is shod, by periodic removal through clipping by the farrier. In general terms, the growth rate of the wall is said to progress at approximately 7mm/month, taking 9-12 months for the whole toe to grow out (Kainer 1987).

Butler and Hintz (1977) acknowledge that the 'rate of hoof growth is of vital importance since it often affects the usefulness of the horse', but Geyer and Schulze (1994) and Zenker *et al* (1995) prefer to express the effect of growth rate on the 'renewal time' of the hoof wall because individual hoof wall length can vary, and thus renewal time for the hoof may be of more practical significance than absolute growth rate. Renewal time, measured in days, is the total time taken for complete dorsal hoof wall replacement from CB to BB.

Manipulation of hoof horn growth rate can have practical implications for veterinary treatment and farriery in terms of growing out a lesion, or for producing sufficient horn for nailing into. A knowledge of hoof horn growth is also an important research pre-requisite for meaningful sampling of like material for comparative testing (Reilly *et al* 1996).

There is misuse of the terms 'growth' and 'growth rate' in the literature. Growth rate has been used where growth has been calculated or observed (Butler and Hintz 1977, Buffa *et al* 1992), and the two terms have been used synonymously. They are different variables:

Hoof growth gives an indication of the total amount of material that has been produced at the end of a given period with units of cm or mm (*eg*: Graham *et al* 1994, Dittrich *et al* 1994, Ott and Johnson 1995).

Hoof growth rate is a time dependent variable, and gives an indication of the pace at which that material has been accumulated, and therefore has units of cm/month or mm/day. (*eg*: Geyer and Schulze 1994, Josseck *et al* 1995). The same hoof growth can be achieved by variable periodic growth rates. Hoof growth rate can be given by the slope of a growth curve or by rates that have been calculated on a periodic basis, with growth up to that point in time being divided by the time relating to that period (Geyer and Schulze 1994, Josseck *et al* 1995). Reported growth rates for equine hoof horn are given in Table 1.1.

Table 1.1 Growth rates for hoof wall (mm/day) reported by different authors

Growth rate (mm per day)	Author
Adults	
0.19-0.28	Glade & Salzman (1985)
0.200	Kainer (1987)
0.208	Caulton Reeks (1906)
0.248 (left fore), 0.257 (right hind) (Lipizzaners)	Josseck *et al* (1995)
0.248 (biotin) 0.25 (control) (Lipizzaners)	Josseck *et al* (1995)
0.273 (control) 0.333 (biotin)	Buffa *et al* (1992)
0.286 (Shires & Belgians) 0.143-0.179 (Icelandics)	Geyer and Schulze (1994)
0.287	Knezevic (1959)
Non-adults	
0.384 (fed ad lib), 0.254 (limited feed) (8 months old)	Butler and Hintz (1977)
0.445 (diet including proteinate) (yearlings)	Ott and Johnson (1995)
0.427 (control) (yearlings)	Ott and Johnson (1995)
0.4375 (yearlings)	Graham *et al* (1994)
0.48 (control) 0.42 (biotin) (yearlings)	Dittrich *et al* (1994)

Hoof wall growth rate is approximately 7mm/28 days (Pollitt 1990, Josseck *et al* 1995), taking approximately 9-12 months for capsule renewal, depending on the absolute wall length. Growth rate of the coronary horn varies between individuals and between breeds. Warmbloods, Belgians and Shires have hoof growth of 8mm/28 days though hoof growth of Icelandic ponies is 4-5mm/28 days. The renewal time of the hooves of Warmbloods is approximately 12 months, Shires and Belgians 15 months and 15-20 months for Icelandic ponies (Geyer and Schulze 1994). Growth rate in youngstock is greater (0.25mm-0.38mm/day i.e. 7mm-10.6mm/28 days (Butler and Hintz 1977). The growth rate of coronary horn is often lower in winter (Geyer and Schulze 1994).

Growth rate of the dorsal wall, side wall and quarters is believed to be uniform at about 7mm/month (Josseck *et al* 1995). Since the hoof wall grows evenly from the coronary epidermis and the distance to the ground is less at the heel, the heel is the youngest and most elastic or least stiff part of the wall. This allows heel expansion to occur (Kainer 1989) as discussed in Section 1.2. Gender has no effect on hoof growth (Butler and Hintz 1977).

When nutrition is inadequate for bodyweight gain and height increase in young ponies, Butler and Hintz (1977) found that hoof growth rate was retarded. This finding influenced the selection of skeletally mature ponies in the experimental design for the work in this thesis.

Pollitt (1990) used radioisotopes to investigate hoof wall growth. Using three injections of 35 s methionine at 14 day intervals he was able to record hoof wall growth over a 28 day period. The labelled isotope was incorporated into new horn growth and he was able to demonstrate that the rate of growth on the external surface of the hoof capsule was 22% lower than the rate of growth at the internal surface. It took 280 and 220 days respectively at each site for the wall to grow from the proximal (CB) to the distal (BB) border.

Three intensely labelled zones of new horn growth, corresponding to the incorporation of 'pulsed' 35 s methionine, were recorded beneath the coronary band. However, these bands were not horizontal. Those in the inner wall had moved further distally than those in the outer wall. This suggests that the rate of horn production in the inner wall is greater than that in the outer layer. Alternatively it may indicate that the outer horn is in a compressed state (Pollitt 1990).

1.7.1. Sliding contact mechanism

Cells overlying the dermal papillae divide and complete the process of cornification and keratinisation as detailed in Section 1.4.

The inner layer of the hoof wall, the *stratum internum* is composed of 600 non-pigmented, keratinised primary epidermal laminae, each of which bears 100-150 non-keratinised secondary epidermal laminae (Pollitt 1992). These dovetail with their adjacent counterparts in the laminar corium (Sisson and Grossman 1953, Trautmann and Fiebiger 1957, Weber 1969) (see Figure 1. 17). There is a horn contribution to the inner 'zone' of the SM of the hoof wall from the *stratum germinativum* overlying the secondary laminae. However, how much of a cellular contribution it gives to the wall is not known.

Fully keratinised primary epidermal laminae arise on the inner shoulders of the coronary groove. The primary epidermal laminae of the SI consist of cells that originate from proliferating basal cells overlying the BM, situated at the coronary border and complete their keratinisation as they are pushed distally with the growth of the wall (Trautmann and Fiebiger 1957). Once formed, they take a 6-8 month journey to the BB, sliding distally past the stationary spinous and basal cells of the adjoining secondary epidermal laminae (Leach and Oliphant 1984). How this is achieved remains unknown.

Pellmann *et al* (1997) have suggested that the secondary epidermal laminae are firmly anchored to their basal membrane by desmosomes to maintain the hoof-phalanx bond, which contributes to the "suspensory apparatus of the pedal bone" (Pellmann *et al* 1997). The primary epidermal laminae are assumed to move distally past the stationary cells of the secondary epidermal laminae by breaking these desmosomal connections. It is assumed that these desmosomes, which join the stationary to the moving parts of the epidermal laminae, break and reform in a "staggered ratchet" like manner, so that the keratinised cells, and hence the whole hoof wall, can move distally during growth and yet still support the load. This is known as the "sliding contact" mechanism (Stump 1967, Leach 1980, Budras *et al* 1989 and Pollitt 1992, 1995b). (See Figure 1. 18).

How this mechanism is consistent with the observations of Pollitt (1990) of differential growth of inner and outer zones of the SM is still unclear and is an area of worthy future study.

Figure 1.17: Transverse section of hoof wall (after Douglas 1993)

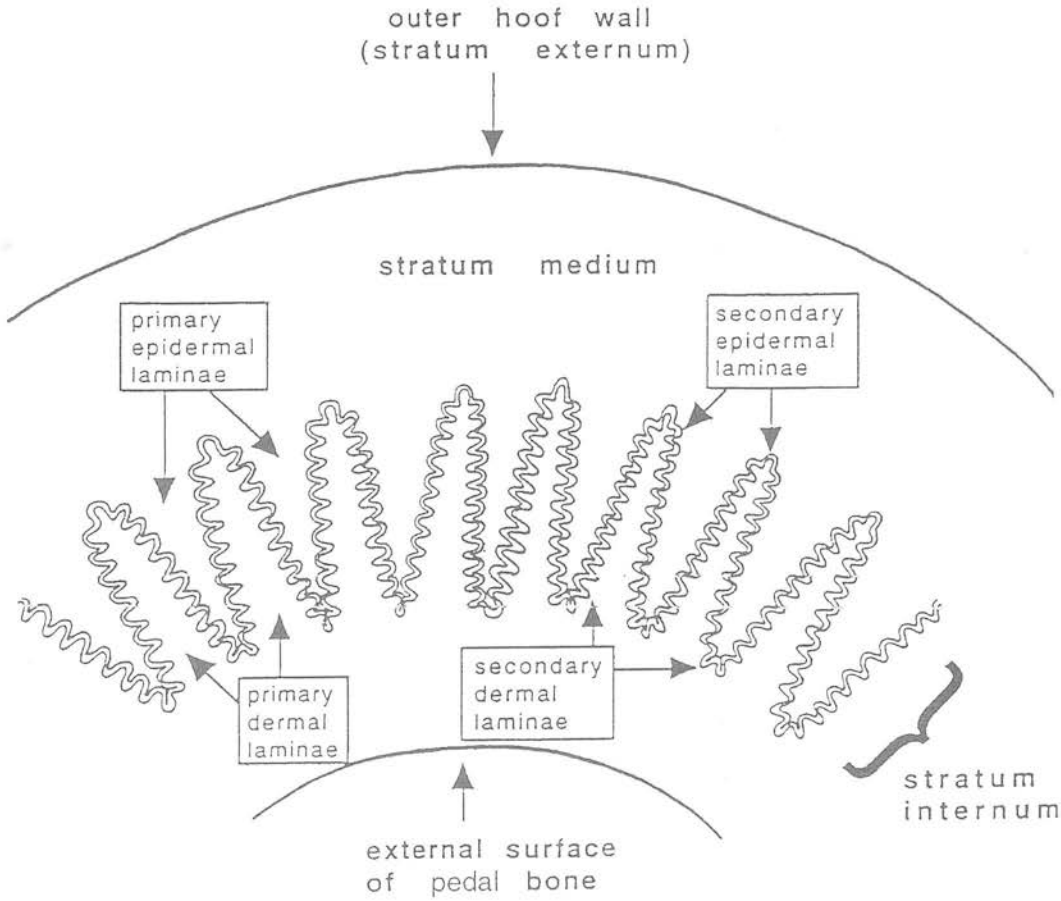
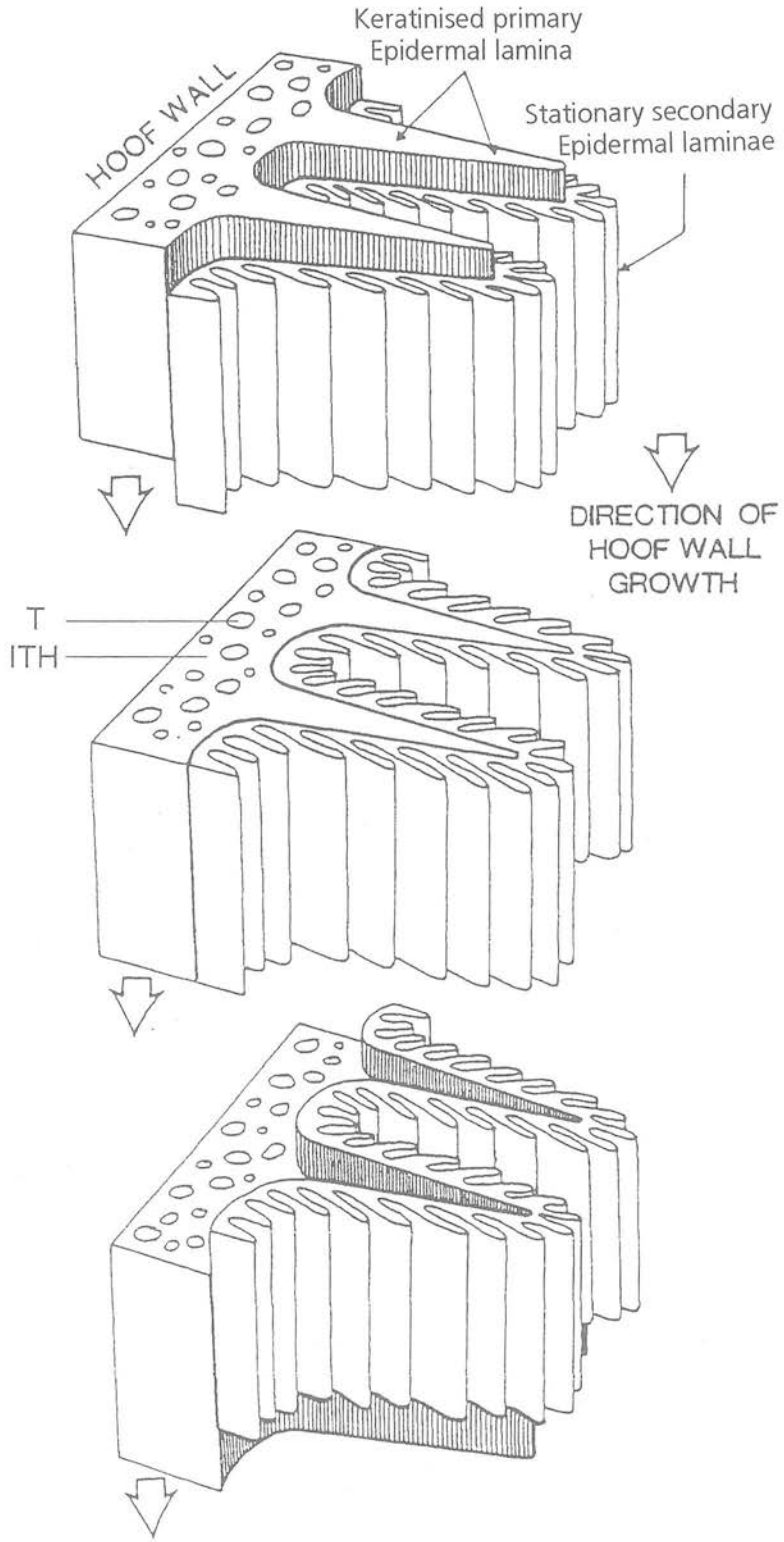


Figure 1.18: The sliding contact mechanism of hoof wall growth (after Pollitt 1995)



Key
T: tubular horn, ITH: intertubular horn

1.7.2. Factors Affecting Hoof Growth Rate

1.7.2.1 Season

Shannon and Butler (1979), Geyer and Schulze (1994) and Ryan, T (1995 - personal communication) have all suggested that there are seasonal differences in horse hoof growth rates, with higher growth rates in spring and summer and decreased growth rates in the winter.

Hahn (1979) and Hahn *et al* (1986) demonstrated that hoof growth in cattle was a cyclical process with maximum growth occurring during the warmer parts of the year. In horses: Shannon and Butler (1979), Ryan, T (1995 – personal communication) and Stashak (1987), in cattle: Vermunt (1990) and Tranter and Morris (1992), and in sheep: Wheeler *et al* (1972) all agreed that growth rate was higher during spring and summer. Ryan, T (1995 – personal communication) reported an average 0.1mm/day increase in growth rate between February and June (from 0.14mm/day to 0.24mm/day respectively).

Clark and Rakes (1982) and Rakes and Clark (1984) suggested that growth rates of cattle claw horn were seasonal and followed variation of daily photoperiod. Vermunt (1990) agreed with this theory. However, Wheeler *et al* (1972) disagreed and suggested that hoof growth was greatly reduced by a lower environmental temperature. Geoghegan and Sandford (1958) found the mean growth rate of sailor's nails in the Arctic to be 0.114mm/day whereas in the temperate coastal waters of Britain, growth rate averaged 0.119mm/day.

Hahn *et al* (1986) suggested that influences on hoof growth rate were multifactorial and that day length or photo period, temperature, management, dietary change and behaviour probably contribute to the cyclical growth pattern of the horse.

Vermunt (1990) proposed that hoof growth is affected by the changes in blood supply to the foot. He suggested that at lower environmental temperature, blood is mainly directed around the core of the horse and the horn-producing cells of the hoof may be inadequately supplied with oxygen and nutrients, which might explain a reduction in horn growth during winter.

1.7.2.2 Genetics

Poor hoof conformation and poor horn quality seem to be inherited in some families of horses and ponies (Eustace 1994). For example, Thoroughbreds tend to have small delicate feet with light walls and a boxy, upright structure according to (Gray 1993). Geyer and Schulze (1994) and Zenker *et al* (1995) found that Lipizzaner horses were affected by hoof wall problems that was assumed to be of genetic origin because of the closed nature of the population.

Evolutionary pressure on the horse may also play an important part in the rates of growth of horse and pony hooves. Anecdotally it is said that breeds that have developed in warmer climates will be more likely to have faster growing hooves. On the other hand, breeds that have developed in wetter, softer areas would have had no such need for fast growing hooves because the rate of natural wear would be slower, and they would therefore have a slower growing hoof wall. These anecdotal arguments persist despite the fact that they disagree with the findings of Camara (1970) and Dietz and Prietz (1981), in cattle, where increased MC% was said to increase the rate of wear of the bearing borders.

This anecdotal argument suggests that horses such as Arabs and Thoroughbreds will have faster growing hooves than horses and ponies native to colder climates such as Britain, for example the Exmoor, Dartmoor and Welsh. The relationship between moisture content and 'wear' of the BB has not been investigated for horses and so remains unresolved. Wear of hoof horn was not considered in this work.

1.7.2.3 Age

Kainer (1987) suggested an age effect in horses with older animals having a slower growth rate of horn.

Shannon and Butler (1979) found that hoof growth rate was greater in young horses than in adults. Similar findings were shown for cattle by Glicken and Kendrick (1977), Tranter and Morris (1992) and Prentice (1973), and in pigs by Johnston and Penny (1989).

1.7.2.4 Sex

Graham *et al* (1994) and Ott and Johnson (1995) suggested sex differences in hoof growth rate with male hoof horn growth rates higher than in females in younger horses, although Butler and Hintz (1977) found no differences in growth between sexes. Schneider (1980) and Schlichting (1987) proposed that sex and breed had no influence on neither growth rates nor wear rates of cattle hoof horn. Miyaki *et al* (1974) however found that hoof moisture content was higher in male than in female horses.

1.7.2.5 Illness

Bean (1963) showed that in man there was a slowing of growth of the nails during illness. Prentice (1973) reported a slowing of growth during the course of a disease, namely lung worm infestation in cattle.

1.7.2.6 Area of the Hoof

For horses, the features of the hoof capsule vary at different sites both radially and proximo-distally. Whether growth of horn at different radial sites around the hoof capsule takes place at different rates is unclear. Caulton Reeks (1906) and Kainer (1987) suggest that this growth is uniform, radially, whereas Geyer and Schulze (1994) reported that the 'palmar/plantar' part of the wall tended to have a lower growth rate in comparison with the dorsal and lateral parts. Josseck *et al* (1995) also found differences in growth rate between midline, lateral sidewall and palmar/plantar sites. Thus, the precise definition of measurement site is important in order to give results of experimental work a context and to allow repetition of study.

Hahn *et al* (1986) found for cattle that growth and wear of the lateral wall were both higher than for the toe. Prentice (1973), also studying cattle, found a significant difference in growth rate between toe, mid wall and heel regions of all claws, with heels growing 40% faster than the toe.

Hahn *et al* (1986) suggested that such different rates of growth can only occur if each tubule in the hoof wall moves independently and one interpretation of this is that the softness of the intertubular horn tissues allows such independent movement. As mentioned in Section 1.7.1 the concept of independent growth of parts of the hoof wall is as yet unexplored.

1.7.2.7 Front and Hind Hooves

It can also sometimes be unclear which measurement(s) from which site(s) on which feet are contributing to the data presented in some work.

Butler (1976) pooled data from one site on all four feet, Graham *et al* (1994) used a midline site and pooled data from the forefeet only. Geyer and Schulze (1994) used three measurement points (dorsal, lateral and palmar) and pooled these data from all four hooves in the first year of study and only from the left fore (LF) and right hind (RH) thereafter. Josseck *et al* (1995) used the same three sites as Geyer and Schulze (1994) and used data from LF and RH only. In addition to defining the site(s) for measurement, it is important to state which feet are contributing to the data set.

Hahn *et al* (1986) found, in cattle, that rear hooves grew faster than front hooves by 9% for dorsal, and 7% for lateral surfaces.

Scott and Butler (1980) suggested that hind hooves grow 12% faster than front feet in foals and 7% faster in weanlings. For older horses front hooves grew 6% faster than hind hooves (Scott & Butler 1980). They postulated that the change in bodyweight distribution upon the hooves, as the animal matures, may explain these observations and suggested that the part of the hoof bearing the most weight will grow the least.

1.7.2.8 Environment

A common environment for the horse is the stable. There is anecdotal evidence that horses kept on shavings have a lower wall moisture content than those kept on straw. Ammonia build-up in stable bedding is said to be detrimental to horn (Eustace 1990). However, the way it is detrimental and which properties of the horn it affects have not been elucidated. Albarano (1993) and Mulling *et al* (1994a) have conducted experiments in cattle where pieces of horn have been subjected to different environmental contaminants separately such as urine and faeces and a mixture of urine and faeces. The mixture was more detrimental to hoof properties than either of the components (Albarano 1993). Thus, the stable or barn environment in which the animal is kept and the way that urine and faeces can accumulate may affect hoof horn properties and it is important to allow for this in experimental design.

Other factors that have been speculated to influencing growth rate include: heart rate, level of exercise, level of metabolism, peripheral blood flow, physical condition, weight distribution or pressure on the hoof, circulating hormones, soil type, neurectomy, irritation or massage of the sensitive structures, shape and angles of the hoof, hereditary and congenital factors and individuality of the horse. However, few of these have been scientifically proven and therefore sound conclusions cannot be drawn from such speculation.

1.7.2.9 Farriery

Farriery is probably the single most important factor that can influence the hoof wall in the horse. Eustace (1994) has reviewed the effects of chronic bad farriery on the hoof wall and the effects of lack of medio-lateral foot balance.

Eustace (1994) suggested that chronic bad farriery leads to stresses in the hoof capsule which eventually results in fracture lines, for example, leaving the toes long usually leads to sagittal hoof cracks.

Continued use of shoes which provide insufficient cover at the heels commonly results in horizontal cracks at the heel quarters which grow down and cause the heel horn beneath the crack to break off.

Longstanding, medio-lateral foot imbalance can result in both quarter cracks appearing and the twisting of the hoof capsule around the long axis of the limb.

All these problems are caused by bad farriery and foot imbalance. In the normal foot, the hoof wall at the toe is parallel to the hoof wall at the heels, and the medial hoof wall is normally slightly more upright than the lateral wall. The angle the hoof wall makes with the ground at the toe should be the same as at the pastern (Colles 1986). This is the ideal for both fore and hind feet even though the dorsal hoof wall angle is ideally and naturally, different at 45° for fore and 50° for hind feet (Stashak 1987).

Any form of poor nailing in which the nails are driven into the wall and exit only a short distance above the shoe will eventually cause the best of feet to crack and split. Nails should be driven into the white line and emerge a third of the way up the wall.

The farrier should trim the hoof to try and correct foot balance and therefore minimise hoof wall problems.

These factors were responsible for the decision to trim animals in the trial for medio-lateral balance only once, at the beginning of the trial, and for the animals to be left unshod.

1.7.3. Nutrition

The earliest report of a nutritional influence on hooves was by Fleming (1871, cited in Butler 1976). The only published controlled and double blind nutritional study of hoof horn using horses to date has been by Zenker *et al* (1995).

Despite this, nutrition is often now being recognised as an important factor in growth of healthy horn with a normal structure (Kempson 1990) but relatively little is known about the effect of nutrition on the growth and composition of the equine foot (Butler and Hintz 1977). Previous nutritional studies have been criticized for lacking experimental controls by Cuddeford (1991), Slater & Hood (1997) and by Buffa *et al* (1992). Apart from the early work of Butler & Hintz (1977) there has been an obvious lack of experimental control in horse hoof studies.

Different authors have suggested that dietary factors exert a keratinogenic effect (Ryder 1973, Kempson 1987, 1990). It has long been known that diet affects the coat of an animal and that conversely, poor nutrition in the young can permanently retard hair follicle development in sheep (Ryder 1973). The loss of pigment and the growth of straight hair has been recorded in sheep suffering from copper deficiency. Zinc and iron (enzyme cofactors) deficiencies have also been found to cause hair loss in sheep and goats (Ryder 1973).

Ryder (1973) stated that the ultimate nutritional factor in determining wool growth was the availability of essential amino acids at the site of the hair follicle. Injections of sulphur-containing amino acids directly into the abomasum in sheep, thus avoiding microbial processing, increased hair growth by 3-4 times (Reis 1989). Obel (1948) established the hoof's high requirement for methionine and cysteine. Kempson (1990) reported an improvement in horn structure following zinc and methionine supplementation. It has been proposed that biotin has an effect upon keratinisation processes in pigs (Tagwerker 1983, Geyer and Tagwerker 1986), cows (Reilly and

Brookes 1990) and the horse (Kempson 1990, Geyer and Schulze 1994, Josseck *et al* 1995 and Zenker *et al* 1995). The rationale for these proposals must be that nutritional factors conveyed in the blood stream are being incorporated into, or are influencing, the manufacture of the hoof wall.

1.8 Nutrient Supply to the Hoof Wall

The hoof wall, in common with all epidermal structures, is dependent upon the underlying dermis for nutrition as shown in Section 1.3. The epidermis is an avascular layer, (Mettam 1896) and it has no neural supply (Sisson and Grossman 1953). Hence the energy for mitotic activity within the *stratum germinativum*, and the nutrients for the metabolic processes associated with the formation of the *stratum corneum* have to be provided by diffusion across the basal membrane from the vascularised dermis (Leach 1980, Bolliger 1991).

To ensure adequate nutrient supply, the coria are highly vascularised and the modification of the dermis into dermal papillae and laminae not only increase the interface for mechanical attachment between dermis and epidermis, but also increases the surface area available for diffusion (Pollitt 1994). Acrylic injection and corrosion casts of the vasculature of the hoof reveal that the capillary network extends into both the dermal papillae and the lamellae (Pollitt 1990) thereby reducing the diffusion distance to the metabolically active epidermal layers.

The processes of keratinization and cornification has an energy demand as well as a demand for other keratinogenic nutrients. What these nutrient requirements are, however, has been little studied. From studies of other keratinous systems such as hair and wool there are assumed to be four main classes of keratinogenic nutrients. Obel (1948) established the hoof's high requirement for the sulphur-containing amino acids methionine and cysteine, and it is largely assumed from research into other keratinous systems such as for hair growth, and optimal functioning of the hair follicle, that an adequate supply of nutrients, including vitamins, minerals, oxidisable substrates and amino acids are required (Galbraith 1998).

Whilst it is known from studies in other epithelial tissues that certain nutrients are important for the keratinization process, there is very little literature on the effects of specific nutrients on the keratinization process in the hoof wall of the horse. Reilly

(1995) remarked that many of the assumptions for the hoof have come from a body of work carried out in the 1950s for other highly keratinized systems which were then of economic importance, such as wool, and which stimulated a stream of research investment at that time. More recently information has been derived from research into cashmere (Galbraith 1998) and human hair (Philpott *et al* 1990). The latter studies have provided information about the effects of epidermal growth factors and hormonal influence on hair production, for example the relationship between iodine intake and thyroid hormone production, and nutritional influence on gene function (Clarke and Abraham 1992, Gurney *et al* 1994).

Broadly, the four categories of keratogenic nutrients are energy, protein (including amino acids), minerals/trace elements and vitamins. A list of recommended daily allowances for them is given in Table 1.2.

Table 1.2 Daily Nutritional Requirements for a 450-500 kg Horse

Nutrient	Resting Requirement for Maintainance	
	Total Requirement	Supplementary Requirement
Digestible Energy (Kcal)	18	-
Digestible Protein (Kg)	0.4	-
Calcium (g)	24	9
Phosphorus (g)	18	4.5
Salt (g)	25	25
Magnesium (g)	5	0.9
Potassium (g)	60	-
Copper (mg)	45	10
Iron (mg)	400	600
Manganese (mg)	100	240
Zinc (mg)	150	240
Cobalt (mg)	5	2.5
Iodine (mg)	1	5
Selenium (mg)	2.5	2.0

After Putnam (1986)

18.1 Energy

Calorific intake has a direct effect upon just about every metabolic pathway and physiological function in the body (Jackson 1996).

A ready supply of oxidizable substrate is required to provide energy for the synthesis of protein and other components of dividing cells, for example, in the hair

follicle and, following differentiation, for the synthesis and deposition of protein in cells of the hair cortex and inner root sheath (Galbraith 1998).

Butler and Hintz (1977) reported that hoof wall growth was 50% greater in *ad libitum*-fed ponies than in feed-restricted ponies. Butler and Hintz (1977) also concluded that the level of protein intake did not affect hoof growth, only the total dietary intake did with young ponies on *ad libitum* feed achieving more growth than those on restricted intakes. However, Jackson (1996) has pointed out that, since the experimental diets of Butler and Hintz (1977) were not isonitrogenous, the effects of energy alone cannot be totally partitioned out. In studies investigating hoof growth it is therefore important to control for the effects of energy and protein.

Energy metabolism and protein metabolism are closely linked. Energy is required for protein metabolism, but energy is also available from protein catabolism. Thus it is important to try and separate this interrelationship by ensuring that protein concentration in the diet is not limiting. Reis (1989) concluded that amino acid supply and not energy is the limiting factor in sheep wool growth. The only circumstance in which energy may be limiting is if protein is supplied in excess and thus there maybe insufficient dietary energy input to catabolise it. More recently, Galbraith (1998) has reported that raw fibre yields and fibre diameter of mohair in Angora goats (Shahjalal *et al* 1992) were increased by, and more sensitive to, changes in the supply of dietary protein, than changes in dietary energy.

A linear response in wool growth to energy supplementation in diets which increased in energy supply from 0.5 to 2.0 x maintenance requirement was recorded in sheep by Marston (1948). Approximately 25% of retained nitrogen was diverted to wool production at twice maintenance, indicating the high priority given to hair deposition in these animals. Protein-energy malnutrition has also been shown to result in reductions in hair diameter and in the number of follicles present in the skin of human subjects (Sherertz and Goldsmith 1991).

1.8.2 Protein

Improved protein nutrition has been shown to increase wool growth in sheep, not only by stimulating protein deposition in differentiating keratinocytes, but also by increasing their rate of initial mitosis (Galbraith 1998). The question of whether amino

acids have a role other than provision of substrate in stimulating mitosis and protein deposition in the hair follicle however, remains to be answered (Galbraith 1998).

Thus, the major nutritional limitation to wool growth is the amount and the composition of amino acids available to wool follicles (Ryder 1973) since wool, hair and hoof will continue to "grow" even on energy limited diets.

Maximum rates of wool growth are attained on maintenance intakes of energy with about 150g of ideal protein available for intestinal digestion, so protein level and type is more influential on wool growth than energy (Galbraith 1998). Protein can be given *per os*, however, elegant experiments in sheep that have had amino acids infused directly into the abomasum, thus avoiding microbial breakdown in the rumen, have shown that the essential amino acids and sulphur-containing amino acids (methionine, cysteine and lysine) are particularly important for wool growth in that they were preferentially and directly taken up into the keratinisation process (Rogers *et al* 1989). Wool and hoof proteins are rich in cysteine and an adequate supply of this amino acid, or methionine to convert to cysteine (see Section 1.9.3.1), is needed by the follicles/papillae to sustain growth (Obel 1948, Eckfalck 1990).

Jackson (1996) found that the hoof wall is composed of 94% protein on a dry matter basis. The amino acid composition of the hoof wall features the sulphur-bearing amino acids methionine, cystine and cysteine. These are involved in cross-linking in proteins such as wool and keratin as discussed in Section 1.4.2. The characteristic crimp in wool is due to cross-linking, which involves these sulphur bearing amino acids.

Vies (1964), Rosenburg *et al* (1957) and Tyson (1950) used gelatin as a protein source and prescribed it for the treatment of brittle finger nails in human beings. However, Reis (1989) stated that gelatin is actually deficient in major amino acids.

There have been conflicting reports on the effect of gelatin on the hoof. Watson (1967) reported that gelatin increased hoof growth and quality. Rosenburg and Oster (1955) reported no increased growth rate in human nail but reported an improvement in 'quality'. In one case the feeding of gelatin increased hoof specific gravity but not hoof tensile strength or amino acid composition (Goodspeed *et al* 1970). Following these reports, Butler (1976) investigated further and concluded that

gelatin did not significantly affect hoof growth or 'quality' as assessed by compressive strength, although there was a trend towards inhibition of hoof growth (Butler 1976, Butler and Hintz 1977).

1.8.3 Amino Acids

Bull (1971) stated, for cattle, that adequate nutrition should be provided in the ration when the hoof is growing fastest i.e. in the spring, in order to maintain a supply of essential elements especially sulphur and sulphur-bearing amino acids at the growth site.

In a study directly aimed at investigating hoof nutrition, Obel (1948) established the hoof wall's requirement for methionine by showing that methionine was differentially taken up by the hoof wall *in vitro*. It is known that an important function of methionine is the provision of cysteine via the trans-sulphuration pathway. Methionine can also be catabolised via the transamination pathway.

Trans-sulphuration involves the transfer of the sulphur from methionine to serine, resulting in cysteine biosynthesis and methionine is 100 percent efficient as a precursor of cysteine. The reaction between cysteine and cystine is freely reversible such that both compounds are equal in furnishing cysteine bioactivity for the support of protein synthesis.

In sheep a specific role for methionine has been found in controlling wool growth as a probable methyl donor or a precursor for polyamine production via S-adenosyline thionine (Reis 1989).

Recent studies by Grosenbaugh and Hood (1993) suggest, however, that whilst methionine is incorporated into the keratin molecules, it probably plays a relatively insignificant role in the structural stability of the hoof wall. Cysteine appears, however, to be preferentially incorporated into the maturing cornified structure and may be an essential component of the final cornification process. Similarly, wool is not rich in either methionine (0.5% by weight) or lysine (3% by weight) but is high in cystine (9-10% by weight) (Mercer 1961).

More recently it has been found that the provision of methionine, lysine or cysteine to sheep do not affect the amino acid composition of wool but they do affect the composition of the IFAPS, the high sulphur-containing and high tyrosine-

containing proteins (Galbraith 1998). This agrees with Grosenbaugh and Hood (1993) for equine hoof horn.

1.8.4 Minerals and trace elements

The major elements that have a role to play in the keratinisation process are copper, zinc and calcium.

The role of minerals and trace elements in bovine foot disease was investigated by Van de Kerk (1970) who attempted to relate soil, crop, and hoof horn levels of calcium, potassium, magnesium, sulphur, copper and zinc on a large number of Dutch farms. He was unable to demonstrate any differences between farms with and without hoof disease, or the levels of minerals in soils and crops, nor any convincing differences in mineral content of hooves that were and were not diseased. He did, however, appreciate the inter-relationships, antagonisms and synergisms between minerals summarised in Figure 1. 19, which must be accounted for during dietary and experimental design in experiments which aim to investigate the effects of dietary supplementation.

1.8.5 Vitamins

There are four key vitamins that have been shown to be critical in horse nutrition in terms of effects on athletic and reproductive performance. These are vitamin A, vitamin E, folic acid and biotin and other B vitamins (Putnam 1986). A full understanding of the requirement for each has not been ascertained.

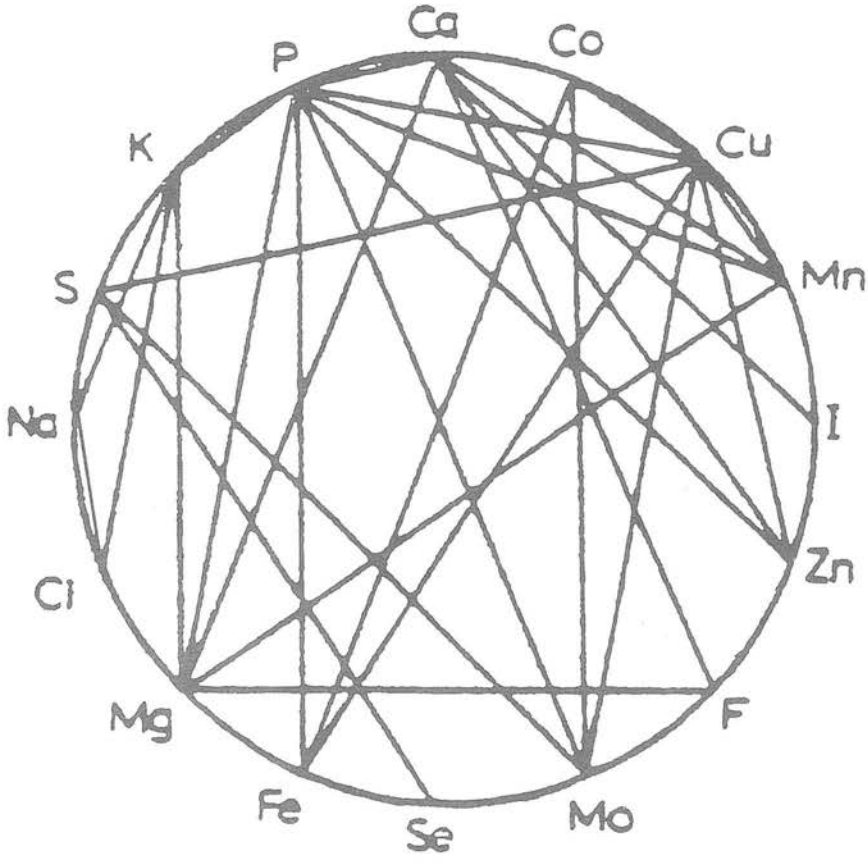
There are 10 other vitamins which are also essential micronutrients, but performance is not so critically dependent on them as on the four key vitamins (Putnam 1986). The 10 other vitamins, though essential, are not primarily related to performance. An inadequate supply of any one can eventually lead to listlessness, inappetence and poor performance (Putnam 1986). The other 10 are: vitamin B_6, vitamin B_{12}, vitamin D_3, vitamin K, thiamine, riboflavin, niacin, pantothenic acid, choline and ascorbic acid. A list of recommended daily allowances for them is given in Table 1.3 (Putnam 1986). The possible effects of each of these in the keratinzation process in the hoof is not known.

Table 1.3 Daily Vitamin Requirements for a 450-500 kg Horse

Vitamin	Resting Requirement for Maintenance	
	Total Requirement	Supplementary Requirement
Vitamin A (iu)	50,000	50,000
Vitamin D_3 (iu)	5,000	5,000
Vitamin E (iu)	550	500
Vitamin K (mg)	5	5
Vitamin B_6 (Pyridoxine) (mg)	100	15
Vitamin B_{12} (Cobalamine) (mg)	2.5	0.2
Vitamin B_1 (Thiamine)	150	24
Vitamin B_2 (Riboflavine)	150	24
Pantothenic Acid (mg)	150	30
Folic Acid (mg)	100	50
Niacin (mg)	250	48
Choline (mg)	2,000	600
Biotin (mg)	1.5	1

After Putnam (1986)

Figure 1.19: Interactions between minerals and trace elements (after Van de Kerk 1970)



Again, the amount of research that has directly set out to ascertain the effect of vitamin manipulation on hoof wall characteristics is sparse. However, driven by the potential for commercial economic return, the information that is available relates to human skin and to hair follicles. Hair follicles are metabolically-active tissues which have complex requirements for both micro- and macronutrients to support structural and functional activities. Sherertz and Goldsmith (1991) have reviewed nutritional influences in human skin, many of which include effects on hair follicle characteristics. In addition to the metabolic role of these nutrients, emphasis has been placed on effects produced by deficiencies, including those due to vitamins and minerals.

1.9 Biotin

Biotin is a water soluble B group vitamin (Uvarov *et al* 1971). It is an essential co-factor in glucose and fat metabolism and it can have profound effects on other pathways by its influence on many other intermediaries (Whitehead 1981). It is essential for growth (Tagwerker 1983) and maintenance of epidermal tissues (Geyer and Tagwerker 1986). It is readily absorbed after oral administration and its plasma kinetics are well described for the horse, (Josseck *et al* 1995, Lindner *et al* 1992). It is not stored in the body (Buffa *et al* 1992) and is safe to feed as it is not toxic even in large doses (McDowell 1989) as it is excreted via the kidneys in excess. Its lack of toxicity is a major reason for its use in equine feeding trials aimed at assessing the effects of dietary nutrient manipulation on hoof horn changes.

1. 9. 1 Discovery and Source

In 1901 Wilders was attributed with discovering a factor called 'bios' which was essential for growth i certain strains of yeast. Subsequent analysis of this factor by Kogl and Tonis (1936) revealed the complex nature of bios. Three different stereo-isomer types of bios (t, -re and neutral) were established and type 'IIB' corresponded to a substance 'Biotin' isolated by Kogl and Tonis (1936) from egg yolk. Furthermore Gyorgy (1941) established that this fractionate was identical to vitamin H (from the German for hair, 'hur,' or skin, 'haut'), which Gyorgy (1931) obtained from rat liver extracts. Whitehead (1988) stated that the isolate known as biotin was found to be identical to coenzyme R which serves as a growth and respiratory factor in some bacteria.

1.9.2 Formula

Kogl and Tonis (1936) reported the empirical formula for biotin methylester was $C_{11}H_{18}O_3N_2S$ which was confirmed by du Vigneaud *et al* (1942). The complete structural determination of biotin was established by du Vigneaud *et al* (1942) as C_{15} hexahydro-2-oxy-1H-thieno[3,4-d]imidazole-4-pentanoic acid. This is shown in Figure 1. 20. Harris *et al* (1943) conducted the first synthesis. Only the (+) stereo isomer of biotin has significant biological activity.

1.9.3 The role of biotin

The above-mentioned extracts were discovered to counteract dermatoses and hair loss in animals and rats in particular, that were fed high doses of egg white (Gyorgy 1931). Egg white contains the protein which binds biotin within the gut, thus making the animals deficient in biotin because of its non-availability.

Biotin is essential for growth (Tagwerker 1983), food utilisation, (Whitehead 1988) maintenance of epidermal tissues (Geyer and Tagwerker 1986) and normal bone development and reproduction (Whitehead 1988). Putnam (1986) explained that biotin is an essential co-enzyme in many pathways, but particularly those liberating stored energy and those concerned with protein metabolism. Biotin is essential in the horse's body for the production of fatty acids, glycogen and proteins Putnam (1986).

In this way, biotin-dependent reactions are involved in both gluconeogenesis and fatty acid synthesis and biotin affects protein synthesis through its influence on the nature and rate of ribonucleic acid formation (Anon 1972, 1984).

The full biochemical role of biotin is still not fully undestood (Anon 1972, 1984). However its metabolic role as an essential co-factor in enzymatically controlled reactions involving carboxylation and decarboxylation is well documented (Moss and Lane 1971).

The four major biotin-containing enzymes in higher organisms are propionyl-coenzyme A-carboxylase (PCC), pyruvate carboxylase (PC), β-methylcrotonyl-CoA carboxylase (MCC), and acetyl-CoA carboxylase (ACC). ACC is a cytosolic enzyme, whereas the other three are mitochondrial enzymes.

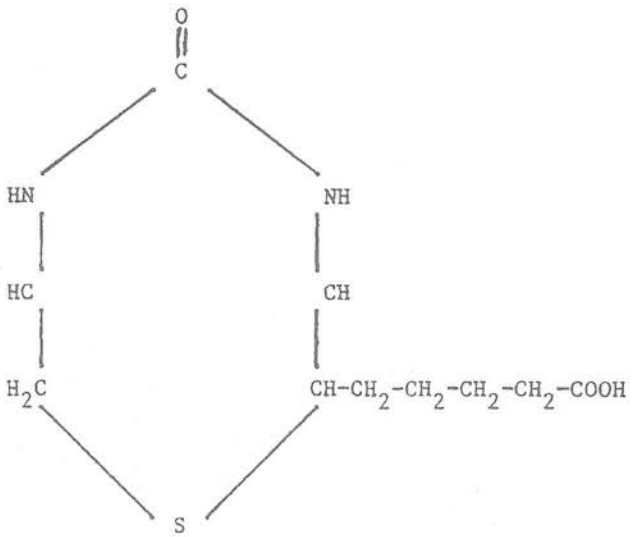
Each of the biotin-dependent carboxylases catalyzes an adenosine triphosphate (ATP)-dependent CO_2 fixation reaction, and in doing so, biotin functions as a CO_2

Figure 1.20: The chemical properties of biotin (after ANON 1972)

Nomenclature:

Biotin is cis-Hexahydro-2-oxy-1H-thieno(3,4)-imidazole-4-valeric acid.

Structural Formula:



Empirical Formula: $C_{10}H_{16}N_2O_3S$

Chemical Properties:

In the pure state biotin is a white crystalline powder which melts, with decomposition at approximately 230°C. It is soluble in water and in alcohol, but is insoluble in organic solvents. It is heat stable, resistant to mild acids and alkalis but is destroyed by strong acids or alkalis.

carrier on the surface of the enzyme. In all carboxylases, biotin is covalently linked to the ε-amino group of lysine. The role of biotin enzymes in intermediary metabolism is shown in Figure 1. 21 (Aurbach 1989). ACC is recognised to be the regulatory enzyme for lipogenesis and hence is required in membrane genesis (Sarasin 1994). PCC is essential for the generation of oxaloacetate, the maintenance of the tricarboxylic acid cycle (Nakano *et al* 1982), and gluconeogenesis in the liver and the kidney. It is also present in lipogenic tissues (eg liver, adipose, lactating mammary gland and adrenal gland) and participates in fatty acid synthesis (Ballard and Hansen 1967). PCC and MCC are required for further metabolism of certain amino acid residues.

1.9.4 Other Functions of Biotin

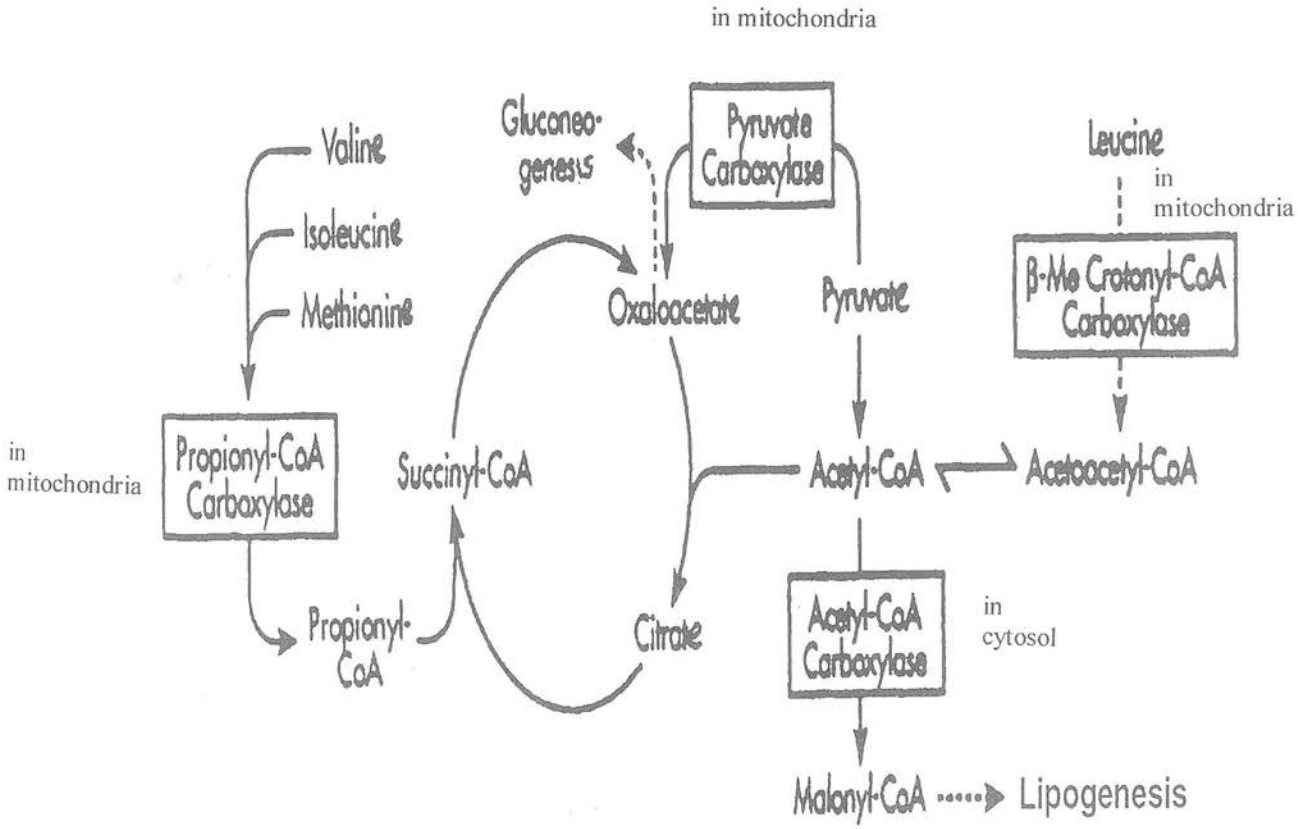
Earlier work on the intracellular fractionation of biotin, in different tissues of rat and chicken, indicated that a significant amount of biotin was associated with the nuclear fraction (Dakshinamurti and Mistry 1963a,b). However, Sarasin (1994) stated that there are no biotin carboxylases in the nucleus, and suggested a function for nuclear biotin other than as a co-enzyme in the biotin-containing carboxylases.

1.9.5 Occurrence and Requirements

Biotin occurs widely in natural foodstuffs (Bonjour 1977). However, even in its richest sources, the absolute concentration of the vitamin is very low. Good sources of biotin are liver (100μg/100g), kidney and yeast (100-400μg/100g) and egg yolk and some vegetables (60μg/100g). Meat, fruit, cereals and bread products show a low biotin content (5-10μg/100g) (Documenta Geigy 1968). Natural biotin exists in its free form in vegetables, fruit and milk or in animal tissues, ligated to proteins which are either enzymes or special binding proteins (Friedrich 1988).

Another source of biotin for human beings and animals is the enteric synthesis of this vitamin by bacteria (Bonjour 1977). Some of the biotin-synthesizing bacteria have been identified in human gut. The extent and significance of this enteral synthesis in overall biotin turnover is not yet known. Since minute amounts of biotin are known to be adequate to support the bodily functions of animals, it has been assumed that requirements are met by natural biotin intakes. In general, daily

Figure 1.21: The role of biotin-containing enzymes in intermediary metabolism (after Aurbach 1989)



requirements are met by the natural biotin content of food and by intestinal biosynthesis of this vitamin (Sarasin 1994).

1.9.6 Intake, Blood Levels and Excretion

Only biotin in its free form i.e. not bound can be absorbed by living organisms (Friedrich 1988). Biotin bound to protein is split off by biotinidase in the gut and is absorbed in the proximal small intestine. The mechanism of transport depends on the animal species: active transport occurs in the hamster and mouse whereas diffusion occurs in rat, rabbit and guinea pig. There is an absorption efficiency of 50% (Friedrich 1988).

Only small amounts of biotin are stored in the liver and the brain. Any vitamin absorbed in excess of the requirement and storage capacity, along with vitamin metabolites (eg bisnorbiotin), are excreted in the urine, whereas unabsorbed vitamin or enterically synthesised vitamin is found in faeces (Bonjour 1977).

1.9.7 Availability to the horse

Whitehead (1988) stated that biotin is widely distributed in a variety of plants including grasses and cereal crops, however it is usually bound to other molecules which can significantly effect its bioavailability. The availability of biotin in most cereals, with the notable exception of maize is very low (Anderson and Warnick 1970, Anon 1972, 1984).

Microbial synthesis of biotin occurs within the rumen of cattle and sheep and is reported to provide a readily available source of biotin to them (Anon 1972, 1984). However, biotin synthesis in the lower GI tract of animals with simple stomachs is considered to provide little available biotin as research has failed to demonstrate absorption from the large intestines (Anon 1972, 1984). Whether hind gut microbial biotin synthesis in the horse is similarly unavailable is not known (Lewis 1995), as controlled studies have not been published on the dietary requirement for biotin in the animal in relation to that supplied by large intestinal synthesis (National Research Council 1989). Moore-Cocanougher (1997) has argued that little of this potential supply of bacterially-derived biotin is actually available to the horse.

With the exception of maize, much of the biotin in cereals is unavailable (Anon 1972, 1984). Lewis (1995) states that the availability of biocytin (i.e. protein-bound biotin) depends upon the digestibility of the protein to which it is bound.

Biotin deficiencies within the diet therefore depend on the content of feed ingredients, the natural form in which biotin occurs, and the degree to which the horse can utilise biotin produced by intestinal bacteria.

Whitehead (1988) stated that the trend seen in ration formulation, particularly for housed livestock such as poultry, cattle and pigs, combined with the increased performance potential of modern strains, has resulted in significantly reduced biotin intake per unit of production.

Concentrates are now replacing significant proportions of forage/fibre in the diet of horses and this may lead to a decrease in biotin intake, especially in the competition horse, for which McGreevy *et al* (1995) has reported reduced grazing times. In an animal whose athletic performance is being pushed, as Putnam (1986) and Jackson (1996) have pointed out (Section 1.8.6) this may lead to an increased requirement for biotin. If these same animals are producing problematic hoof horn then biotin supplementation has been suggested in order to correct the hoof wall defects.

It is thought that the normal horse has a natural dietary requirement of 2-3 mg of biotin per day (Putnam 1986). Horses with poor hoof wall horn generally do not have a significantly different plasma biotin concentration from their good-footed contemporaries (Geyer and Leu 1988, Johnston 1991). Normal plasma biotin concentration is from 200-300 ng/l (Geyer and Leu 1988, Johnston 1991). According to Jackson (1996) there have been numerous reports in the literature which have attributed improvement in hoof wall histological properties to supplementary biotin. In these studies, supplementary biotin levels of 15-20 mg per day have had positive effects on histological characteristics of the hoof wall tissue (Jackson 1996). Additionally according to Jackson (1996), some have reported an increase in the tensile strength of hoof wall. Jackson (1996) also reported that some horses appear, subjectively, to respond to biotin supplementation while others would "not respond if fed ten pounds of biotin per day". This lack of response in some cases may be due to lack of absorption and subsequent utilization.

1.9.8 Biotin effects on keratinisation

Biotin appears to be of particular importance in controlling the production and deposition of keratins (Whitehead 1988).

Biotin deficiency causes an impairment in protein synthesis as Dakshinamurti and Litvak (1970) have shown that this is due to inhibition of RNA synthesis. Mistry and Dakshinamurti (1964) and Achuta Murthy and Mistry (1977) concluded that biotin deficiency depressed pyruvate oxidation and thus reduced ATP required for other anabolic pathways.

Mercer (1961) stated that enzymes which catalyse carboxyl activation of at least two amino acids have been identified. Such activated groups form an essential intermediate stage in protein synthesis. Given that biotin is known to be an important prosthetic group in certain carboxylation reactions, then it can be argued that biotin may play a vital role in keratin synthesis. Sarasin (1994) has confirmed the earlier work of Fritsche (1990) and Fritsche *et al* (1991) to show that biotin does alter the expression of certain keratins in *in vitro* studies.

1.9.9 Biotin and the Hoof

Interest in biotin's role as a dietary supplement to manipulate hoof horn production followed reports by Brooks *et al* (1977) which suggested that foot lesions found in commercial pigs corresponded with those reported by Cunha *et al* (1946) in experimentally induced, biotin-deficient pigs.

Cuddeford (1991), Reilly (1995) and Slater and Hood (1997) have all called for controlled experimentation in the study of nutrient effects on hoof horn characteristics. May (1989) concluded that 'most of the claimed treatments for poor quality hoof horn were 'empirical and anecdotal'. This is still true today and Reilly *et al* (1998a) commented that "heed must be taken of the recent call for more rigour in setting up experiments involving equine animals which aim to assess the effects of a therapy". If this is not done then "therapy becomes lore; medical myths have arisen this way" (Rossdale 1997). This is in danger of becoming the case in this field of study. Unfounded attempts at therapy for hoof horn problems will perpetuate medical myths (Reilly *et al* 1998a). More recently, controlled studies investigating the effects of supplementary dietary biotin on hoof horn production have been carried out by

Buffa *et al* (1992), Dittrich *et al* (1994), Josseck *et al* (1995) and Zenker *et al* (1995). The results of these experiments are discussed later in this section.

It has been proposed that biotin has an effect upon keratinisation processes in pigs (Tagwerker 1983, Geyer and Tagwerker 1986), cows (Reilly and Brookes 1990) and the horse (Kempson 1990, Buffa *et al* 1992, Geyer and Schulze 1994, Dittrich *et al* 1994, Johnston 1991, Josseck *et al* 1995 and Zenker *et al* 1995).

Comben *et al* (1984) investigated the influence of supplementary biotin on the gross appearance of the equine hoof capsule, because similar horn defects had been seen in horses that had been seen in pigs. These defects, described as 'thin friable horn', had been controlled by the addition of biotin to the diet in pigs (Brooks *et al* 1977, Comben *et al* 1984, Simmins and Brooks 1985).

In a Review, Vermunt and Greenough (1995) reported that Reilly and Brooks (1990) had studied the effect of supplementary dietary biotin on horn hardness and growth and wear rate in dairy cattle. The biotin supplemented group produced significantly harder horn ($p<0.05$) in the centre of the abaxial wall after 13-15 weeks of treatment. There was no significant effect of the treatment on horn growth rates, but there was a trend for the biotin supplemented animals to have a greater net horn growth, indicating that they experienced less wear (Vermunt and Greenough 1995).

Geyer and Schulze (1994) stated that improvements in equine horn quality at various sites in the hoof wall, in response to biotin supplementation, is dependent upon horn produced at the coronary band reaching the site. However, Simmins and Brooks (1985) observed increases in hoof hardness in pigs that could not be interpreted in terms of horn renewal, thus concluding that hoof hardness could be influenced by biotin levels after the horn tissue was formed. These findings are consistent with subjective observations made by Comben *et al* (1984) in the horse.

Geyer and Tagwerker (1985) reported that lameness in pigs was relieved following high doses of biotin before the lesions had grown out.

1.9.9.1 Biotin deficiency

In addition, there are species specific deficiency disorders. Pigs fed biotin-deficient diets, for example, suffer from loss of weight, indigestion, dermatitis and lesions of the skin and feet (Geyer and Tagwerker 1986). Dogs especially lose hair and suffer from severe dermatitis (Bonjour 1977). Although enough biotin is thought to be produced by the microbial flora in the rumen of cattle, claw problems in cows can be decreased with supplementary dietary biotin. After biotin administration, their claws get harder and show fewer fissures (Cooke and Brumby 1983).

There are no reports concerning biotin deficiency in horses. Biotin supplementation studies in the horse have been limited and have, with the exception of Johnston (1991), Buffa *et al* (1992), Zenker *et al* (1995) and Jossecks *et al* (1995), mainly relied upon subjective assessment of small population samples. Biotin treatment improves the quality of the hoof (in terms of tensile strength and histological scores) without stimulating horn growth in pigs according to Geyer and Tagwerker (1986) and Johnston and Penny (1989). However, Marston (1948) suggested that biotin deficiency did not effect the process of keratinsation in sheep but did however alter the process of cellular division and maturation of the proliferative cells in the lower epithelial strata.

1.9.9.2 Biotin effects on hoof growth rate

Different researchers have investigated the effect of biotin upon hoof/claw growth rate. Neither Johnston and Penny (1989) nor, Geyer and Tagwerker (1986) working with pigs, nor Reilly and Brooks (1990) with cattle, detected any significant effect upon growth rates following supplementation. However Macoun (1982) (cited by Comben *et al* (1984)), Bains (1985), Ribeiro (1987) and Buffa *et al* (1992) all reported increased hoof growth in the horse with biotin supplementation. Factors such as weight, age and breed were reported to influence hoof growth rate in cattle (Vermunt and Greenough (1995).

Fritsche (1990) and Fritsche *et al* (1991) investigating the effect of biotin upon *in vitro* keratinocyte cell lines reported that pharmacological doses of biotin enhanced both cellular proliferation and differentiation. Fritsche (1990) stated that these changes were initiated by a hormone-like mechanism leading to an increase in mRNA of certain cytokeratins.

The addition of pharmacological doses of biotin (1 μmol and 100 μmol) to these *in vitro* keratinocyte cell lines caused specific increase in cytokeratins, which are normally induced upon terminal differentiation of epidermal cells *in vivo*. (Fritsche 1990, Fritsche *et al* 1991). The expression of cytokeratins occurring in stratified epithelia, independent of differentiation, were not affected. The results show that biotin directly stimulates the differentiation of epidermal cells, which may provide an explanation for the observed therapeutic changes in growth rates by the above authors.

Conversely, Geyer and Leu (1988) and Zenker *et al* (1995) observed no significant difference in growth rate between biotin supplemented and control groups of horses. Reilly and Brooks (1990) found no change in hoof growth rate in cattle supplemented with dietary biotin and Dittrich *et al* (1994) reported a reduced hoof growth rate of hoof horn in horses receiving biotin, compared with controls. ($P<0.01$). Thus, results are equivocal about the effects of biotin supplementation on hoof growth rate.

1.9.9.3 Subjective anatomical effects of biotin on the gross hoof capsule

Brooks *et al* (1977) reported a 28% decrease in hoof lesion incidence following biotin supplementation over a 6 month period in pigs, and concluded in the same way as Tagwerker (1983) that biotin supplementation had induced a 'hardening of tissue'.

Since replacement gilts had a lower incidence of lesions with biotin supplementation, Brooks and Simmins (1980) concluded that supplementation of the diet of breeding sows with biotin from an early stage of development made a significant contribution to the maintenance of their horn integrity.

Triebel and Lobsiger (1979) concluded that biotin may be beneficial to young stock destined for breeding, as a consequence of hardening of the claw. Johnston and

Penny (1989) suggested that supplementation more readily prevented rather than cured hoof lesions.

Such observations in pigs suggest that biotin not only has a therapeutic effect in reducing hoof capsule lesions, but that it also exerts a prophylactic effect upon the hoof capsule. It can therefore be argued that biotin may in some way affect the process of horn production and or hoof structure and/or maintenance of hoof integrity in the normal hoof capsule.

Comben *et al* (1984) observed improvements in both hoof defects and in general capsule conformation following biotin supplementation at 15 mg / 350 kg BW horses over a six month period. This was based upon subjective analysis of individual case studies with friable horn. Likewise Schulze and Scherf (1989) who supplemented horses with similarly poor quality hoof horn at similar rates, for at least 9 months, reported that, the condition was cured in 24 horses and improved in the remainder.

However Dittrich *et al* (1994) supplementing 1-2 year old Thoroughbreds at 40 mg/350kg BW recorded improvements after only 3 months of biotin supplementation.

Josseck *et al* (1995), using a semi-quantiative system for scoring 'alterations' to hoof horn histology, evaluated changes in hoof horn condition in response to biotin supplementaion over a 9 month period in 42 Lipizzaner horses at the Spanish Riding School. This study was not only conducted under controlled experimental conditions, but represented the first project in the horse that avoided using a small population sample (Reilly (1995), Slater and Hood (1997)), and in this way group data for horses was obtained.

Josseck *et al* (1995) reported that the group supplemented with biotin significantly improved hoof horn histological features in terms of histological scores compared with both pre-trial scores, and compared with the control group, after 9 months of supplementation. Moreover further improvements occurred in the following 5 months and were then maintained over three subsequent years of evaluation.

Geyer and Schulze (1994) reported similar improvements in macroscopic horn assessment following biotin supplementation to horses with brittle feet. However, reduction in the level of supplement, or its complete withdrawal, resulted in deterioration in horn condition (Geyer and Schulze 1994).

1.9.9.4 Subjective microscopic and ultrastructural effects

Kempson (1987) published an observational account of the microscopic and ultrastructural characteristics of the hoof wall in two case studies of horses with brittle feet. The first study made observations from distal hoof clippings using the scanning electron microscope (SEM) and reported the presence of 'holes' and a complete loss of tubular structure within its SE. However the SM and SI were said to exhibit a "well consolidated, normal structure with good inter-squame (keratinocyte) attachment". Supplementation with biotin over a 9 month period in this case resulted in a gradual improvement in "gross structure" and the "restoration of tubular horn structure" (Kempson 1987).

However, the horse in the second case study had been supplemented with 20 mg of biotin daily for the previous 6 months without improvement. A SEM examination also revealed no disruption to the tubular structure of the SI but normal tubular structure was reported to be completely absent from the SI. Hoof horn keratinocytes were also noted to have separated from each other, with major planes of separation parallel to the long axis of the wall (Kempson 1987).

Biotin supplementation, augmented on this occasion by limestone and additional dietary protein introduced at a later stage, resulted in improvements in gross structure over 9 months. Kempson (1987) suggested that, in this case, it was the calcium from the limestone addition that improved the horn. Examination of hoof structure at 9 months, again from clippings, revealed well-defined tubules and improved inter-keratinocyte adhesion. Kempson (1987) stated cohesiveness was not as intimate as in "good horn". However, no comparative observational account of "good horn" was given.

Kempson (1987) referred to observations on thirty-three other horses with brittle feet. Of these, thirty-one were reported to have loss of tubular structure within the hoof wall. Twenty had previously received biotin supplementation without

improvement. Improvements in hoof structure were claimed following elevation of calcium and protein levels in the diet. Again in all these cases a concurrent control group was absent.

The use of distal clippings from the bearing border for the assessment of horn characteristics or horn production raises several issues. The bearing border is subjected to mechanical impact and abrasion, together with biochemical and microbial challenge (Budras, Scheil and Mulling 1998). It therefore makes sense to use another site for sampling, which avoids biomechanical change at the clipping site and which can be sampled with a known history in terms of growth rate. The use of the MDC site allows this.

The small number of horses (n=2) evaluated under field study conditions, by Kempson (1987) and the lack of a concurrent control, led to questions being raised regarding the reliability of this report with regard to the influence of biotin by Cuddeford (1991) and Slater and Hood (1997).

In an improved study in pigs, which this time used matched controls, Kempson *et al* (1989) supplemented youngstock with dietary biotin to 80 kg slaughter weight. Differences were observed between treatment and control animals in the microscopic and ultrastructural characteristics of the SM. The observations of Kempson *et al* (1989) were that intertubular horn had greater "integrity", keratinocytes were more "tightly packed", and tubules were more "clearly defined" in the biotin-supplemented group and the coronary epidermis of the biotin treated group was said to be 'more ordered and of better structure'. Kempson *et al* (1989) concluded that these differences were due to biotin-induced changes to the keratinization and cornification processes between the two groups.

Biotin supplementation of women with brittle nails has also been reported to give subjective improvements in both macroscopic nail condition and cellular density and arrangement (Colombo *et al* 1990).

Budras and Geyer (1989) observed ultrastructural changes in the membrane coating material, which is responsible for cellular adhesion (Budras and Bragulla 1990), in hoof horn samples taken from horses reported to have poor hoof quality.

Geyer and Schulze (1994) suggested that biotin supplementation may have a direct influence upon the cell membrane, or MCM, such that the binding substance between the cells is improved or that premature decay is avoided (Josseck *et al* 1995). Johnston and Penny (1989) suggested that biotin and zinc in someway improves the binding capacity of the inter-tubular horn in pigs.

1.9.9.5 Biotin effects on tubule marrows

The initial observations of Geyer *et al* (1988) of a decrease in tubule marrow size with biotin supplementation has been subsequently supported by results from other workers such as Geyer and Schulze (1994) and Dittrich *et al* (1994).

Geyer and Schulze (1994) observed 'histological alterations' within the proximal part of the outer hoof wall in three groups of horses of differing breed type. Hoof wall block samples were removed from the outer SM using a miniature disc saw and hammer and chisel. Geyer and Schulze (1994) interpreted these "alterations" as being indicative of degenerative horn cells, noting that 'histological alterations' corresponded with 'enlarged' medullary spaces.

Although each group was reported to contain horses with brittle feet along with individuals with good quality horn, it is unclear which criteria were used to define normal sized marrow and the distribution of such an "alteration" within each group.

Biotin supplementation was conducted over a 3 year period with each group divided into a treatment and control population. Geyer and Schulze (1994) reported that supplementation resulted in improvements in histological appearance, i.e. reduced medullary size (as measured by a semi quantiative scoring system based upon a four level numerical ranking system from 0 'no alteration' - 3 'severe alteration') compared with the control. These improvements were observed after 8 months, and continued until month 15, after which this condition was maintained for the remainder of the trial. After 16 months of supplementation tubule marrows were reported to be smaller, yet some degenerative cells were still evident. Quantitative analysis of the proportion of hoof horn taken up by tubule marrows, for which the method was unstated, revealed a reduction from 25% prior to supplementation to 12%, but the authors neglected to state whether this was the percentage area of marrow compared

with the tubule area as the reference point, or compared with the whole hoof horn area as the reference point.

Zenker *et al* (1995) reported the presence of medullary enlargement in the outer zone (in 12% of horses) although normal ranges were not cited. Zenker *et al* (1995) also suggested that cortical decay could weaken the tubular cortex and the same authors observed a loss of tubular structure within the inner zone. This finding was similar to that seen by Kempson (1987).

1.9.9.6 Biotin effects on tubule density

Kempson *et al* (1989) made objective measurements of tubular density (TD) in pigs, that is, the number of tubules per mm^2 of hoof horn. However, the method by which the results were derived was not given. Mean tubular density was higher in the biotin-supplemented group of pigs at 144 tubules per mm^2 compared with 96 tubules per mm^2 for the controls. Mean connecting horn width , adjacent to the laminae, was also not as wide in the treatment group, at 100μm, compared with the control group at 144 μm.

Since this report, Dittrich *et al* (1994) have also reported an increase in tubule density effect in four horses on a high dose only of supplementary biotin (40mg/day).

The process by which tubule density increases is not addressed in the literature. Such an increase might suggest that biotin increases the number of dermal papillae. Alternatively, Bragulla, H (personal communication 1993) suggested that a lack of ' verdammenn' or tubule 'fade out' in which the tubules lose their regular cellular arrangement, may in part explain the mechanism. Unfortunately, Zenker *et al* (1995) did not provide supportive evidence from comparative observations of the inner zone from proximal horn samples where loss of tubule structure had been reported from distal clippings.

Although the study was conducted on a small population sample of three groups of four horses Dittrich *et al* (1994) observed differences in marrow diameter, tubular density and intertubular 'appearance' between biotin-treated and control groups. They also suggested a possible dose dependent response to biotin with TD effects, for example, only seen at the higher of 3 dose rates; 10 mg, 20 mg and 40 mg total dose per horse per day.

Whereas the control and 10 mg groups were of 'similar' appearance, marrow diameter decreased progressively with increased rates of supplementation; tubular density increased significantly in the 40 mg group; and inter tubular cohesion increased with increased levels of biotin.

Dittrich *et al* (1994) stated that reported changes in microscopic parameters in a 40 mg biotin-supplemented group of horses coincided with improvements in the subjective assessment of the hoof capsule's gross anatomical features of the hoof capsule such as "appearance" and "integrity".

Dittrich *et al* (1994) concluded that biotin supplementation improved the 'strength' of the hoof capsule not only by increasing tubular density, as suggested by Mauske (1972), but also by decreasing the marrow diameter. However no mechanical testing was conducted to support this hypothesis.

1.9.9.7 Biotin effects on mechanical properties of hoof horn

Webb *et al* (1984) concluded that there would appear to be a rational biomechanical basis for the findings by others that biotin reduces lameness.

For a number of keratins a correlation exists between stiffness and the amount of high sulphur-containing and glycinetyrosine proteins in the matrix of the keratin fibres (Bendit and Gillespie 1978). Webb *et al* (1984) referred to a personal communication from MacRae (1982) who proposed that biotin may bring about improvements in strength by affecting the amount and proportion of matrix proteins. This proposal together with the suggestion of Buffa *et al* (1992), that biotin may act as a coenzyme involved in the biosynthesis of keratins from cytoplasmic polypeptide, may be justified in view of the findings of Fritsche (1990), Fritsche *et al* (1991) and Sarasin (1994) who found a change in the expression of keratin proteins with *in vitro* supplementation of biotin to keratinocyte cell cultures.

Fritsche (1990) and Fritsche *et al* (1991) reported that pharmacological doses of biotin to keratinocyte cell lines enhanced both cellular proliferation and differentiation. Pharmacological concentrations of biotin (1 μmol and 100 μmol) caused specific increase in cytokeratins, which are normally induced upon terminal differentiation of epidermal cells *in vivo*. (Fritsche 1990, Fritsche *et al* 1991). The expression of cytokeratins occurring in stratified epithelia independent of

differentiation were not affected. The results show that biotin directly stimulates the differentiation of epidermal cells, which may provide an explanation for the observed therapeutic changes in growth rates and other reported effects of nutritional doses of biotin on the hooves of farm animals.

Bragulla, H (personal communication 1993) considered that the MCM was the most important factor in determining cellular cohesion and that biotin supplementation may affect the composition of it. Mulling *et al* (1994b) had concluded that the chemical composition, amount and distribution of MCM is responsible for intercellular cohesion. Hence it is decisive in determining the strength of the *stratum corneum* (Mulling *et al* 1994c).

The amount of MCM is thought to be influenced by the size of the intercellular space and the presence of MCM in bubble-like dilatations within it (Mulling *et al* 1994c). In hard horn, this space is relatively narrow and regular with occasional dilatations containing MCM. However in soft flexible horn, this space is irregularly wide with large dilatations containing large amounts of MCD (Mulling *et al* 1994c). These observations are similar to the ultrastructural observations recorded by Kempson (1991a,b) in horses reported to have poor quality horn. Evidence of an association between this feature and altered mechanical properties however, is still lacking.

Neither Brooks *et al* (1977), Kempson *et al* (1989) in pigs, or Comben *et al* (1984) or Kempson (1987) in horses, conducted mechanical tests to confirm their suggestion that hoof hardness increased following supplementation. Webb *et al* (1984) demonstrated in pigs significant quantitative increases in hardness and compressive strength, parallel to the horn tubules within the lateral wall, with biotin supplementation, whilst hardness values decreased in the heel. However, compressive strength at the leading edge of the hoof wall remained unchanged (Brooks and Simmins (1980), Webb *et al* (1984)). Brooks and Simmins (1980) suggested that no change was evident at this site because it was already at maximum hardness.

Geyer and Schulze (1994) and Zenker *et al* (1995) investigated the inter-relationship between microscopic characteristics, tensile strength at failure of coronary horn, hoof quality and the effect of biotin supplementation over time in

horses. Both studies suggested a possible association between microscopic hoof characteristics and tensile strength, although no statistical relationship was demonstrated.

In both studies, pooled results were calculated from a combination of MDC, lateral and medial sites, and samples were stored at 20°C at 65% relative humidity for 96 hrs prior to testing.

Geyer and Schulze (1994) recorded significant differences in hoof horn tensile strength between those horses displaying "histological alterations", such as medullar enlargement and micro-cracking, with those showing "unaltered horn". Tensile strengths were lower in histologically altered horn. These findings are in broad agreement with Zenker *et al* (1995) who reported reduced tensile strength in specimens with microcracks and medullary enlargement in the outer zone of the proximal horn.

However the effect of biotin supplementation upon the relationship between tensile strength and microscopic characteristics is difficult to ascertain. Geyer and Schulze (1994) observed improvements in tensile strength with biotin supplementation after 8 months in those individuals initially identified as having "altered" horn. Strength values approached those of horses with "unaltered" horn after 15 months. These increases coincided with descriptions of decreased medullary diameter and a decreased medullar area fraction Geyer and Schulze (1994).

Conversely Zenker *et al* (1995) observed no significant differences in tensile strength within or between the groups after 19 months supplementation, despite histological improvement in terms of micro-cracking having been observed in the biotin treated group at this stage of the trial. Furthermore a significant increase in tensile strength in the control group was recorded after 23 months. However significant differences between groups ($P< 0.05$) and a barely significant difference within the biotin group $P = 0.06$ were recorded after 33 months. Differences were, however, noted in the number of samples considered unsuitable for testing, which fell progressively in the biotin group throughout the trial, whereas numbers remained constant in the control. Zenker *et al* (1995) stated that this represented improvements in hoof quality as a result of biotin supplementation. However neither the criteria for exclusion nor detailed histological evaluations of such samples were detailed. The

absence of improvement in either histological assessment (after 19 months) or in tensile strength (after 33 months), given a hoof renewal time of 11-12 months, are difficult to reconcile.

However, Tagwerker (1983) observed a similar discrepancy in lesion resolution time between piglets and sows, which could not be explained merely in terms of different horn renewal rates. Geyer and Tagwerker (1986) suggested that the sow's greater bodyweight might interfere with the healing process. Impaired hoof function, resulting from the presence of hoof lesions, would lead to corium tissues being subjected to abnormal tension and pressure, which would lead to abnormal horn production. This could account for the persistence of medullary enlargement after 15 months of biotin treatment reported by Geyer and Schulze (1994), although a genetic predisposition for altered horn cannot be discounted.

Definitive conclusions regarding the effect of biotin supplementation on these aspects of hoof horn properties cannot be drawn given the apparent contradictory findings reported in these two studies.

Webb *et al* (1984) stated that it is inappropriate to consider the hoof as a 'monolithic homogeneous structure'. Hence different parts of the hoof may well respond differently to supplementation. Webb *et al* (1984) suggested that improvements in mechanical properties of horn at certain locations within the hoof wall may not occur if it is either already at its optimal level, or is limited by factors which biotin does not effect.

1.10 Conclusions and Aims of thesis

From the literature review it can be seen that there is little quantitative understanding of the anatomy of the hoof wall SM in any species and of the functional role of its components. There is scant evidence of whether or not nutrition has any effect on the production of hoof horn or its subsequent properties. This lack of information prevails partly because there has only been a small body of work in the field and partly because some of that work has been poorly designed in scientific terms, through a lack of appropriate controls. In addition, the reported work suffers from a lack of specifically reported methods in terms of measuring objective and well-defined features of the hoof horn capsule at defined sites of horn production. Thus

there has been insufficient controlled data generation and analysis in this field which has made only a modest contribution to the base of knowledge.

Therefore it was decided to conduct a controlled feeding experiment using clinically normal ponies to assess whether supplementation with one manipulated nutrient, biotin, at a single dose level, had any effect on the physiological, anatomical and mechanical properties of newly-produced hoof horn at a specific site on the hoof capsule. Knowing the age of animal and the site and age of hoof horn produced was important in order to minimise the potential confounding factors for experimental work that have been covered in Section 1.7.

Biotin was chosen as the test nutrient of choice in order to assess whether it effected change in hoof horn physiology anatomy and mechanics and because it is not toxic in excess. For the purposes of experimental control and minimising confounding factors, it could be fed as a high dose supplement that would not interfere significantly with the metabolism of other minerals and vitamins as discussed in Section 1.8.

In addition, a recent body of controlled work had appeared in the literature that had raised interesting data about the possible effects of biotin supplementation. With the design of a controlled feeding trial, the results of quantitative analysis of hoof horn samples that had been produced under carefully controlled experimental conditions, could then be compared with published data.

Bragulla, H (personal communication 1993) has suggested that size of tubules may be a more important determinant of horn function than tubular density. The question still remains as to which aspects of the morphometric features of hoof horn; the absolute area measurements of the horn tubule, cortex or marrow or the relative proportion of tubular to intertubular horn is most important in terms of function. This was to be determined in this work by relating morphometry to mechanical testing. The overall hypothesis to be tested was whether or not supplementary dietary biotin at a single dose level had any effect on the physiology, anatomy, and mechanical properties of the hoof horn.

The areas worthy of objective study with regard to possible effects of biotin supplementation were concluded to be: measurement of the *physiological* properties of growth and growth rate of hoof horn, measurement of *anatomical* properties relating to tubule density, absolute area measurements and area fractions of

components of the SM, and measurement of mechanical properties relating to bending stiffness and moisture content of hoof horn.

Aims of the thesis

The aims of this study were to identify normal values for the specific features of the hoof wall listed below, and to determine whether or not biotin supplementation at a single dose rate has any effect on them.

The null hypothesis to be tested was that "Dietary biotin supplementation, at a single dose rate, has no effect on the physiology, anatomy, or mechanics of pony hoof horn".

The specific hoof wall features investigated were:

- growth and growth rate of pony hoof horn (Chapter 2)
- tubule density within the *stratum medium* (Chapter 3)
- absolute area measurements and area fractions of marrow, cortex, tubule and intertubular horn (Chapter 4)
- bending stiffness and moisture content of pony hoof horn (Chapter 5)

The thesis also aimed to identify whether or not there were any inter-relationships between the above quantitative hoof horn parameters (Chapter 6).

The detail of the materials and methods chosen are also explained in the forthcoming chapters as follows:

Chapter 2 gives the experimental design and the method for the measurement of hoof growth and growth rate.

Chapter 3 gives the method by which histological samples were prepared and from which subsequent tubule density analysis took place.

Chapter 4 gives the method by which histomorphometric area fraction measurements were carried out.

Chapter 5 gives the method by which bending stiffness and moisture content of hoof horn was assessed, and discusses the use of a finite element analysis (FEA) technique which was subsequently used to model gross hoof capsule mechanics.

In order to investigate any possible inter-relationship between form and function, univariate correlations of the data from Chapters 2-5 are given in Chapter 6, together with a discussion of the conclusions of the thesis.

The originality of enumerated conclusions from the thesis is given in Chapter 7.

Chapter 8 identifies future research directions.

CHAPTER 2

Growth and growth rate of the hoof wall

2.1 Introduction

The question of whether or not dietary biotin supplementation has an effect on the growth and growth rate of hoof horn is equivocal. Bains (1985) and Buffa *et al* (1992) found that biotin supplementation increased hoof growth, Geyer and Schulze (1994) and Josseck *et al* (1995) found no difference in growth rate following supplementation, and Dittrich *et al* (1994) reported a decrease in hoof growth following biotin supplementation.

Some of the differences in results from previous workers may be attributable to the different doses of biotin used. Buffa *et al* (1992) gave 7.5mg and 15mg of biotin to horses for which body weights were not reported. Geyer and Schulze (1994) gave 20mg to Warmbloods as did Josseck *et al*(1995) to Lippizanners. Dittrich *et al* (1994) gave 10, 20 and 40mg, as single doses to horses for which body weights and ages were not reported. Assuming these amounts were given to 500kg bodyweight (BW) horses, these equate to dose rates of 0.015mg/kgBW for 7.5 mg, 0.03mg/kgBW for 15mg, 0.04mg/kgBW for 20mg and 0.08mg/kgBW for 40mg.

Section 1.7 has illustrated that hoof horn growth and growth rate studies can be confounded by factors such as age, sex and breed of horse, site of reading on the hoof capsule, time of year, nutrient intake and dosage of nutrient given. These factors were taken into account for the design of the experimental work in this thesis.

For this study it was decided to test biotin. This was because of its safety when fed at super-supplementation levels compared with alternative test nutrients such as zinc, copper or methionine which are toxic in excess. In addition, the body of literature on biotin and hoof horn had revealed sufficient questions, and flaws in experimental approaches, to warrant a controlled experimental approach.

2.2 Aims

The aim of this part of the experiment was to investigate the effect of dietary biotin supplementation at one dose rate on hoof growth and growth rate at a specified site on the hoof capsule of all four feet of ponies, in a controlled matched-pair designed feeding trial.

Specifically, the aims of this Chapter are to:

i) explain the experimental design used in the work;

ii) explain the method used for measuring hoof growth and growth rate;

iii) present the results for growth and growth rate and investigate whether there was an effect of biotin supplementation.

2.3 Materials and methods

2.3.1 Experimental Design

Four pairs of ponies were matched for sex, age, weight, size and breed type (see Table 2.1). Two pairs (four animals) were Shetland ponies and two pairs (four animals) were 'pony types' (i.e. cross-breeds). Their previous history was largely unknown but they were selected in a "vetting" based on general signs of health and lack of obvious hoof capsular defects. The ponies were bought from two sources. Prior to purchase, potential pairs were aged from their dentition by two different veterinary surgeons. On arrival the ponies were made to wear permanent headcollars, of one colour, with clearly visible numbers (1-8) written in indelible ink on the sides of the collars. Each pony had an individual identity card written up, together with their headcollar number, and this information was stored on a board in the accommodation as per Home Office requirements. All animals were wormed with Eqvalan™ on arrival and had their teeth rasped. Their feet were trimmed for medio-lateral balance by the Field Station farrier. All eight animals were then loose-housed in a barn on wheat-straw bedding with access to *ad lib* water.

All the day to day husbandry tasks, feeding, watering and bedding up, were carried out by the author, apart from a period of two weeks during the basal period, i.e. after the animals had settled in. The ponies were fed a commercial high-fibre pony cube diet which contained 100 μg/Kg of biotin. The details of the cube diet are given in Table 2.2. This was fed, from individual feed mangers, as a common basal diet for an acclimatization period of 12 weeks prior to the supplementation experiment beginning. This gave a period during which controlled basal hoof growth could take place. No further supplement, nor hay, was fed after the basal period although each of the animals had equal access to eat their bedding. The total daily ration allocated to each individual was calculated on the basis of 0.015kg food fed per kg bodyweight

Table 2.1: Details of ponies in the trial, daily feed intake and daily biotin intake

Pair	Animal	Treatment (T) or Control (C)	Description	Weight (kg)	Age (yrs)	Sex	Mean Pair Bodyweight (kg)	Fresh Weight Feed per day (split into 2 feeds) (kg)	Daily Total Biotin Intake Basal + Supplemented (mg)	Daily Biotin Dose Rate (mg/kg bodyweight)
1	Pony 1	C	chestnut welsh type	245	2	mare	257.5	3.86	0.386 + 0 = 0.386	0.00158
	Pony 5	T	dun welsh type	270	2	mare			0.386 + 30.88 = 31.266	0.1158
2	Pony 2	C	bay welsh type	257	7	gelding	246	3.69	0.369 + 0 = 0.369	0.00144
	Pony 6	T	bay welsh type	235	6	gelding			0.369 + 29.52 = 29.889	0.1272
3	Pony 3	C	chestnut shetland	204	8	mare	208	3.12	0.312 + 0 = 0.312	0.00153
	Pony 7	T	black shetland	212	8	mare			0.312 + 24.96 = 25.272	0.1192
4	Pony 4	C	black shetland	170	12	mare	165	2.48	0.248 + 0 = 0.248	0.00146
	Pony 8	T	black shetland	160	14	mare			0.248 + 19.84 = 20.088	0.1256

Table 2.2: Nutritional specification of the basal diet

Component	Proportion in Diet as Fed
Dry Matter	86%
Energy	9.6 MJ/kg DM
Crude Protein	10%
Oil	2.75%
Fibre	20%
Ash	8.5%
Vitamin A	5800 iu
Vitamin D_3	1000 iu
Vitamin E	9 iu
Vitamin B1	1 mg/kg
Vitamin B2	3.62 mg/kg
Nicotinic Acid	25 mg/kg
Pantothenic Acid	10 mg/kg
Biotin	100 µg/kg
Vitamin B12	18 µg/kg
Vitamin K	1 mg/kg
Manganese	11 mg/kg
Zinc	50 mg/kg
Iron	33 mg/kg
Cobalt	0.25 mg/kg
Iodine	0.4 mg/kg
Calcium	1.2 %
Phosphorus	0.5 %
Copper	13 mg/kg
Selenium	0.2 mg/kg
Molybdenum	1.0 mg/kg

(BW). In order to control for the effect of total energy intake on hoof growth (Butler and Hintz 1977), both animals within a pair received a ration that was calculated for the mean bodyweight for the pair (see Table 2.1). In this way energy, protein, mineral and vitamin intakes were controlled.

2.3.2 Housing and Identification

The animals were initially all housed in one barn and were allowed to run together as one group in the whole of the barn area. However, it soon became apparent that, beyond the initial and expected 'settling in' period, that No 1 (eventually Control pony No 1) was bullying No 8. Since the bullied pony was evidently depressed and spent long periods of time in self-isolation from the others, and did not spend desirable amounts of time feeding, it was decided to house the animals as two seperate groups in this initial period to afford No 8 some protection from No 1. This disrupted the ideal planning of the experiment as this meant that there was a bias in that No's 1 and 8 could not be eventually penned together in the same treatment or control group. The bias was that they could not be both randomly allocated to the same group but had to remain separated. At the end of the acclimatization period the animals were (otherwise) randomly allocated to treatment or control groups in the following way:

Random allocation to treatment (T) or control (C) group was achieved by writing each of the names of a pair on separate pieces of paper and placing all 4 pairs of names, as pairs, face down on a table. One of each pair was then randomly selected. These individuals remained on the basal diet and became the control group. The remaining individuals were fed the treatment diet and became the treatment group.

At the end of the acclimatisation period the animals had to be moved. This was because the barn had to be returned to University farm use. Four other pens were made available, two each in separate barns. To prevent bias due to accommodation, a pair of ponies, consisting of a treatment animal and its matched control, were randomly allocated to each pen. Pairs 2 and 3 were allocated to the pens in one barn and pairs 1 and 4 were randomly allocated to the pens in the other barn. These animals remained in these pens for the rest of the trial and were only taken out for

periodic foot measurements, as described in Section 2.3.4. The conditions in each pen were similar except that one barn faced south and had more incoming light, whilst the other barn faced east. The fact that a matched pair of animals were in each pen was designed to control for any bias by pens within each respective barn.

After the acclimatization period and allocation to T or C groups the ponies were then given colour coded headcollars: the treatment group ones were red and numbered 1-4. The control group ones were blue and numbered 1-4 according to the pairs. This afforded easy identification and was a safeguard for easy identification should any mix up of animals occur. This did not occur throughout the experiment.

This method of identification also proved very satisfactory for the feeding routine:

2.3.3 Feeding

For the first 2 weeks all ponies were fed hay only to bring them all to similar levels of fibre intake and to break them gently into a new feeding regime. The pony cube diet was also introduced on an increasing basis over the first 2 weeks so that at the end of that period all ponies were on the diet only. There were no problems with lack of acceptance of the diet.

After a period of 12 weeks (the 'basal period') with all animals on the basal diet, one of each pair of ponies was randomly allocated (as described above) to continue with the basal diet which then became known as the 'control' diet. The animals fed this diet were known as the control animals. All the individuals of the pairs that were allocated and fed the control diet were known as the control group. The remaining individual in the pair was fed a high biotin-supplemented diet. This became known as the 'treatment' diet. The animals fed this diet were known as the treatment animals. All the individuals of the pairs that were fed the treatment diet were known as the treatment group.

The diets which arrived from the manufacturers in labelled bags were stored on separate pallets in a locked feed shed. This was where twice daily preparation of the animals' feed took place. Great care was taken at all times never to contaminate the control diet with biotin from the treatment diet. A routine was adopted as follows: each animal had an individual plastic feed bowl. Four of the feed bowls were

blue plastic which corresponded to the blue headcollars for the control group. Four of the feed bowls were red plastic which corresponded to the red headcollars for the treatment group. Each of the 2 groups of feed bowls was numbered 1 to 4 and thus corresponded with the number marked on the sides of the red and blue headcollars in treatment and control groups respectively. Thus one bowl remained as an individual's feed bowl throughout the trial. Before feeding commenced, each individual was tethered at its respective corner of the accommodation. This ensured that each individual had access to its own food only and could eat without disturbance. This also meant that individual intakes could be assessed, but this was not required as on all occasions all food was eaten with no requirement for 'weighback'. During the feeding period, 'mucking out' and re-bedding duties were carried out by the author as well as cleaning and replenishment of water bowls. Fresh water was available to all animals *ad libitum* at all times except during tethering for pony cube feeding. Similarly, access to straw bedding was *ad libitum*.

Before preparing feeds the author's hands were washed. Control diets were always prepared first and then fed before treatment diets were fed. The authors hands were then washed before feed bowls were collected in: 'control' bowls first, followed by 'treatment' bowls. As this was a time-consuming procedure, and the daily process of weighing individual's feed requirements according to Table 2.1, increased the chances of mistakes being made, it was subsequently decided to prepare all feed for the week into individually colour coded and marked plastic bags. The same precautions to prevent cross contamination were carried out. The animals were fed each morning at approximately 8 am and each afternoon at approximately 5 pm. Sufficient time was given for the animals to eat up whilst other husbandry duties were carried out. Pairs 1 and 2 were fast eaters. Pair 3 were variable and pair 4 were slow. Since the rate at which pair 4 ate their food was limiting, all bowls remained in place until this pair had finished. This could take up to 40 minutes.

The treatment diet was exactly the same as the basal diet except for the addition of 8mg of biotin per kg of food as fed. This was fed, as was the control diet, on a mean paired weight basis at a rate of 0.015kg of food per kg of BW. The total daily ration was divided to give two equal feeds. The absolute amount of biotin fed to

each individual within the treatment group varied (see Table 2.1) but the dose rate was consistent at 0.12mg/kg BW. Similarly, the absolute amount of biotin fed to control animals maintained on the basal diet varied, but the dose rate to them was 0.0015mg/kgBW (See Table 2.1).

Biotin supplementation continued for a further five months during which time the extent of new hoof growth was measured by the author with records taken by his wife.

2.3.4 Measurement of hoof growth

New horn grows distally from the level of the coronary band (CB). An assessment of growth was given by measuring the distance to which a hot-branded mark ('X'), made on the hoof wall of each foot of each pony, had descended, at given times during the trial, with respect to a proximal reference point in the region of the coronet. The 'X' mark was made with the flat-filed end of a Philips screwdriver which was heated to red hot and placed approximately 1.5mm into the dorsal hoof wall. The definitive feature used as a reliable proximal fixed point was the reference hairline (RH_L) at the coronet. This was revealed by turning back the fringe of hair that normally hangs over the CB.

Both the CB and the RH_L have been used by previous authors as reference points in the assessment of hoof horn growth which is subject to potential measurement error. Geyer and Schulze (1994) used the CB and Butler and Hintz (1977), Graham *et al* (1994) and Josseck *et al* (1995) all used the $R.H_L$. Distances were spanned using dividers, which were then placed against a steel ruler with 1mm divisions which could be used to give measurements to the nearest 0.5mm to improve the accuracy of measurement in this work, but the repeatability of measurements also needed to be addressed.

2.3.5 Repeatability and Reproducibility

Repeatability and reproducibility are the terms used to describe the precision and variability of test methods (British Standards Institute 1987). Repeatability is a measure of the minimum variability and refers to tests performed under conditions that are as constant as possible, for example during a short interval of time, ideally using the same specimens and equipment, with one operator.

Reproducibility is a measure of the maximum variability and refers to tests performed in widely variable conditions with different specimens, equipment and operators (British Standards Institute 1987) and did not apply to this work.

A pilot study established that the repeatability of measurements to the RH_L. was within 1% whereas for the CB it was >4%. Measurement to the CB as the reference point was therefore rejected.

2.3.6 Definition and Justification of Midline Dead Centre

Because of the differences in anatomy at different sites around the hoof capsule reported by Nickel (1938, 1939), Bucher (1987) and Schummer *et al* (1981), and the debate about whether hoof growth rates are the same , or different, at different parts of the capsule, it is essential for growth studies and for morphometric assessment of horn that sampling is standardised at a consistent and accurately defined anatomical location. In this way, differences arising from sample location can be controlled. The sampling site chosen was the Midline Dead Centre (MDC) and was determined as follows:

The MDC is in the sagittal plane of the hoof capsule and was determined by extending an imaginary line which bisected the frog onto the dorsal hoof wall at the bearing border (BB). This point was marked in with chalk. A perpendicular line from this point, following the line of the visible dorsal wall hoof tubules to the coronary band (CB) was marked with chalk before branding. This perpendicular line represented the MDC.

The MDC was selected because it offers several distinct advantages over other locations within the capsule. Primarily it is one of the few sites that can be defined with accuracy, as it is relatively easy to locate. It is therefore readily reproduced between individuals and thus 'inter-sample' consistency is ensured.

In summary the MDC was chosen as the site for measurement for the following reasons:

i) Any nutritional effect, or lack of effect may be different at different sites around the foot and so the area of interest must be precisely defined.

ii) The MDC is the only point about which hoof wall anatomy is symmetrical with equal wall width to either side of it as well as symmetry of laminae. These are

important considerations for subsequent histomorphometry and mechanical testing of horn specimens. Medial and lateral heels have decreased wall thickness (Leach 1980) and different curvatures proximodistally and radially, and do not have the same internal anatomy;

iii) When the horse moves, the foot 'breaks over' about the MDC. This can have many variations, but, in balanced feet, the foot should break over the MDC. Thus horn taken from this site, between individuals, would have grown under known conditions and have been subject to symmetrical stresses. The decision to sample from this site, for reasons of stress symmetry, has since been backed up by the findings of Thomason *et al* (1998).

Initial individual brand marks on the feet of each pony were made below the distal extremity of the periople (XG_0 in Figure 2.1). Each mark was made at a variable point proximodistally on each hoof, between 1.5 to 3.5cms below the RH_L, but the distance from it to the RH_L gave the initial baseline reading for each foot from each animal (RH_LG_0, column 1, Table 2.3). The brandmark was not made any higher as the procedure might have damaged periople as well as horn that was subsequently of interest for use in other tests. New horn growth from the coronary band, G_n, was therefore assumed to equal G_1 minus G_0, the difference in measurements from the hairline (see Figure 2.1).

Initial growth measurements for the allocated treatment and control groups were made for the last twenty-six days of the twelve week basal feeding period prior to biotin supplementation beginning. Subsequent growth measurements were made periodically but on the same day for all animals. Period intervals ranged from 32-45 days and are shown in Figure 2.2.

To avoid bias in a non-blinded experiment, data were collected from one group of ponies first and stored, and then data were collected from the other group (on the same day). In this way direct comparison between treatment and control pairs was not allowed during data collection, and analysis of the data did not take place until some months after the experiment had finished.

Table 2.3: Hoof horn growth data for all ponies by period of trial

Animal and Hoof	RH_1G_0 (mm)	RH_1G_1 (mm)	Period 1 G_n (RH_1G_1-RH_1G_0) (mm)	RH_1G_2 (mm)	Period 2 Cumulative G_n (RH_1G_2-RH_1G_0) (mm)	RH_1G_3 (mm)	Period 3 Cumulative G_n (RH_1G_3-RH_1G_0) (mm)	RH_1G_4 (mm)	Period 4 Cumulative G_n (RH_1G_4-RH_1G_0) (mm)	RH_1G_5 (mm)	Period 5 Cumulative G_n (RH_1G_5-RH_1G_0) (mm)
Control Animals											
Pony 1 LF	19.0	26.0	7.0	32.0	13.0	39.0	20.0	45.0	26.0	51.0	32.0
RF	16.0	21.5	5.5	24.0	8.0	33.0	17.0	38.5	22.5	47.0	31.0
LH	27.5	30.5	3.0	35.0	7.5	45.0	17.5	52.0	24.5	60.0	32.5
RH	24.0	27.5	3.5	32.0	8.0	44.0	20.0	49.5	25.5	57.0	33.0
Pony 2 LF	16.5	23.0	6.5	29.0	12.5	40.0	23.5	46.5	30.0	50.0	33.5
RF	15.0	18.0	3.0	22.0	7.0	34.0	19.0	39.0	24.0	47.0	32.0
LH	20.0	28.0	8.0	33.0	13.0	45.5	25.5	50.0	30.0	58.0	38.0
RH	25.0	30.0	5.0	34.0	9.0	43.5	18.5	50.0	25.0	54.0	29.0
Pony 3 LF	22.5	31.5	9.0	43.0	20.5	52.5	30.0	55.5	33.0	64.0	41.5
RF	23.5	30.0	6.5	35.0	11.5	46.5	23.0	51.5	28.0	53.0	29.5
LH	28.0	33.0	5.0	39.0	11.0	48.0	20.0	58.0	30.0	63.0	35.0
RH	26.5	34.0	7.5	43.0	16.5	56.0	29.5	60.0	33.5	64.0	37.5
Pony 4 LF	18.0	22.0	4.0	26.5	8.5	36.0	18.0	40.0	22.0	47.5	29.5
RF	12.5	16.0	3.5	20.0	7.5	24.0	11.5	29.5	17.0	35.0	22.5
LH	20.5	22.5	2.0	26.0	5.5	31.0	10.5	34.0	13.5	37.0	16.5
RH	21.0	23.0	2.0	25.0	4.0	31.0	10.0	34.0	13.0	39.0	18.0
Treatment Animals											
Pony 5 LF	29.0	34.5	5.5	42.0	13.0	55.5	26.5	61.5	32.5	68.0	39.0
RF	18.5	25.0	6.5	31.0	12.5	42.5	24.0	51.0	32.5	61.0	42.5
LH	26.5	30.5	4.0	36.0	9.5	49.0	22.5	58.0	31.5	65.0	38.5
RH	32.0	37.0	5.0	42.0	10.0	55.5	23.5	63.0	31.0	70.0	38.0
Pony 6 LF	32.5	37.0	4.5	42.0	9.5	53.0	20.5	64.5	32.0	68.5	36.0
RF	16.0	23.0	7.0	31.0	15.0	41.5	25.5	48.0	32.0	52.0	36.0
LH	33.0	38.0	5.0	44.0	11.0	59.5	26.5	64.0	31.0	67.0	34.0
RH	34.0	38.5	4.5	45.0	11.0	55.5	21.5	63.0	29.0	69.0	35.0
Pony 7 LF	23.0	33.0	10.0	41.0	18.0	49.5	26.5	57.0	34.0	65.0	42.0
RF	17.5	24.5	7.0	32.5	15.0	41.5	24.0	48.5	31.0	58.0	40.5
LH	24.0	27.5	3.5	33.5	9.5	43.5	19.5	50.0	26.0	57.0	33.0
RH	24.5	30.5	6.0	39.0	14.5	50.0	25.5	56.5	32.0	66.0	41.5
Pony 8 LF	22.5	27.5	5.0	33.0	10.5	39.0	16.5	44.0	21.5	52.0	29.5
RF	18.0	23.0	5.0	26.0	8.0	29.0	11.0	36.5	18.5	43.0	25.0
LH	21.5	26.0	4.5	30.0	8.5	39.0	17.5	43.0	21.5	51.5	30.0
RH	23.0	26.5	3.5	30.0	7.0	35.5	12.5	43.0	20.0	48.0	25.0

Figure 2.1: Measurement of hoof horn growth and hoof horn growth rate at midline dead centre

Growth is given by: $\overline{RH_LG_1} - \overline{RH_LG_0}$ and is equal to the growth from the coronary band, G_n.

Hoof horn growth rate is given by: $\frac{\overline{RH_LG_1} - \overline{RH_LG_0}}{t_1 - t_0}$

2.3.7 Statistical analysis:

Growth is a continuous variable, notwithstanding the factors affecting it as discussed in section 1.7, and would be expected to have a normal distribution. To test this an n-scores probability (Minitab Corp) was plotted for treatment and control data. A correlation of 0.976 for n=32 (8 horses 4 feet each) was given. This meant that the hypothesis for normal spread of the data could be accepted at a probability of p=0.01. Thus, parametric tests; Students t-test and analysis of variance were used to assess the data using the Minitab software package.

2.4 Results

2.4.1 Hoof horn growth

The data set for hoof growth readings during the course of the trial is given in Table 2.3. The cumulative mean growth of horn from the MDC of the feet of all 8 ponies is plotted by period of trial in Figure 2.2.

The growth of horn for treatment and control groups in the 26 days before supplementation began was compared by t-test. (Period 1 in Figure 2.2). There was no significant difference between the two groups (p=0.62).

By the end of the trial treatment animals had achieved a mean hoof growth at the MDC of : 35.34mm (S.D:± 5.63)

The control group achieved : 30.69mm (S.D:± 6.78)

This difference was significantly different ($p<0.05$) by Student's t-test.

Thus, there was a positive treatment effect on hoof growth after 5 months of biotin supplementation.

2.4.2 Hoof horn growth rate

The slopes of the growth curves (i.e. growth rates), for treatment and control groups were assessed by comparison of regression. The whole data sets contributed to the regression lines and they are shown in Figure 2.3.

The regression equation for control hoof horn growth was:

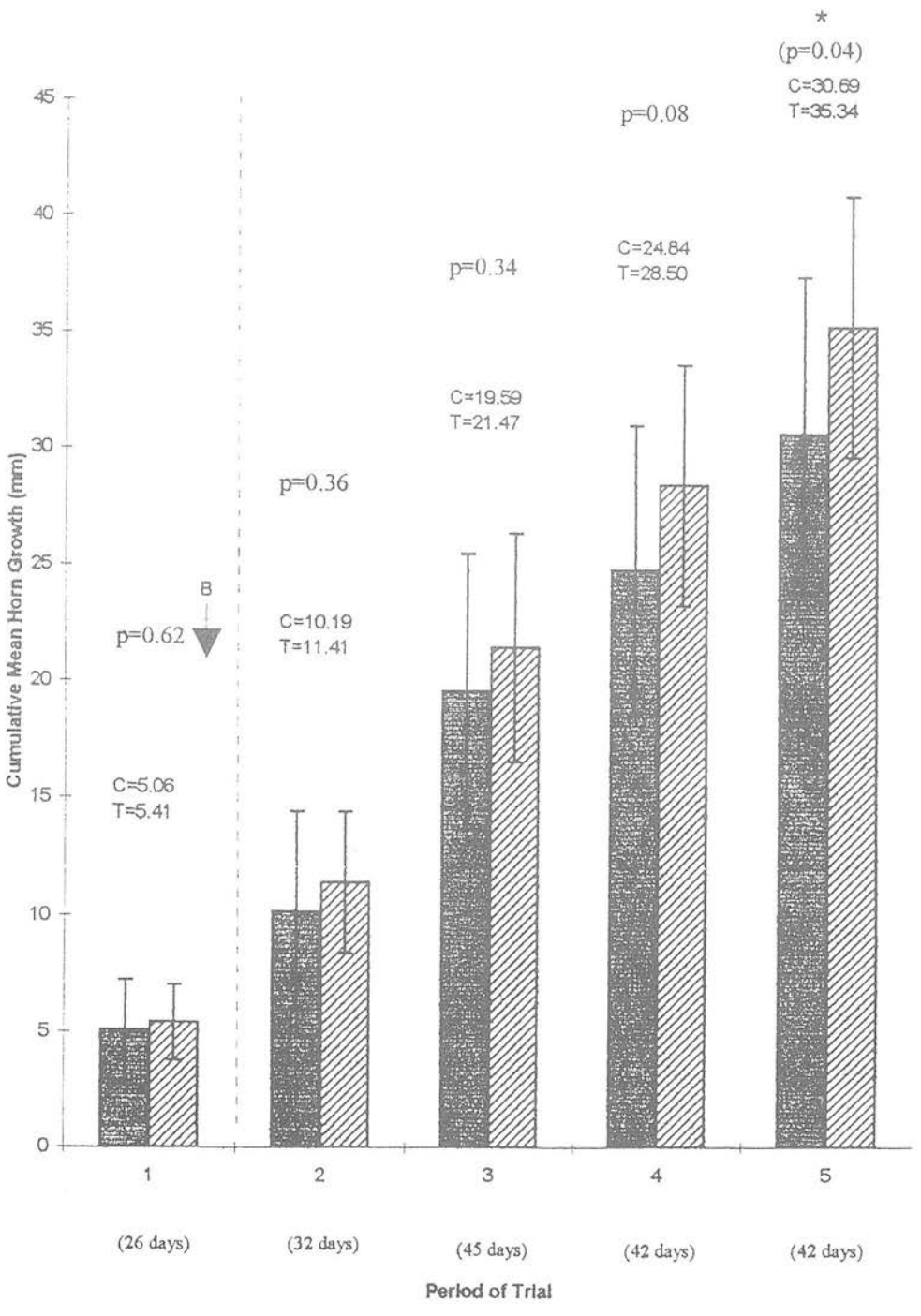
$$y = 6.5906x - 1.6969$$

The regression equation for treatment hoof horn growth was:

$$y = 7.6969x - 2.6656$$

The standard deviation for control hoof growth rate was 1.263 and the standard deviation for treatment hoof growth rate was 1.107.

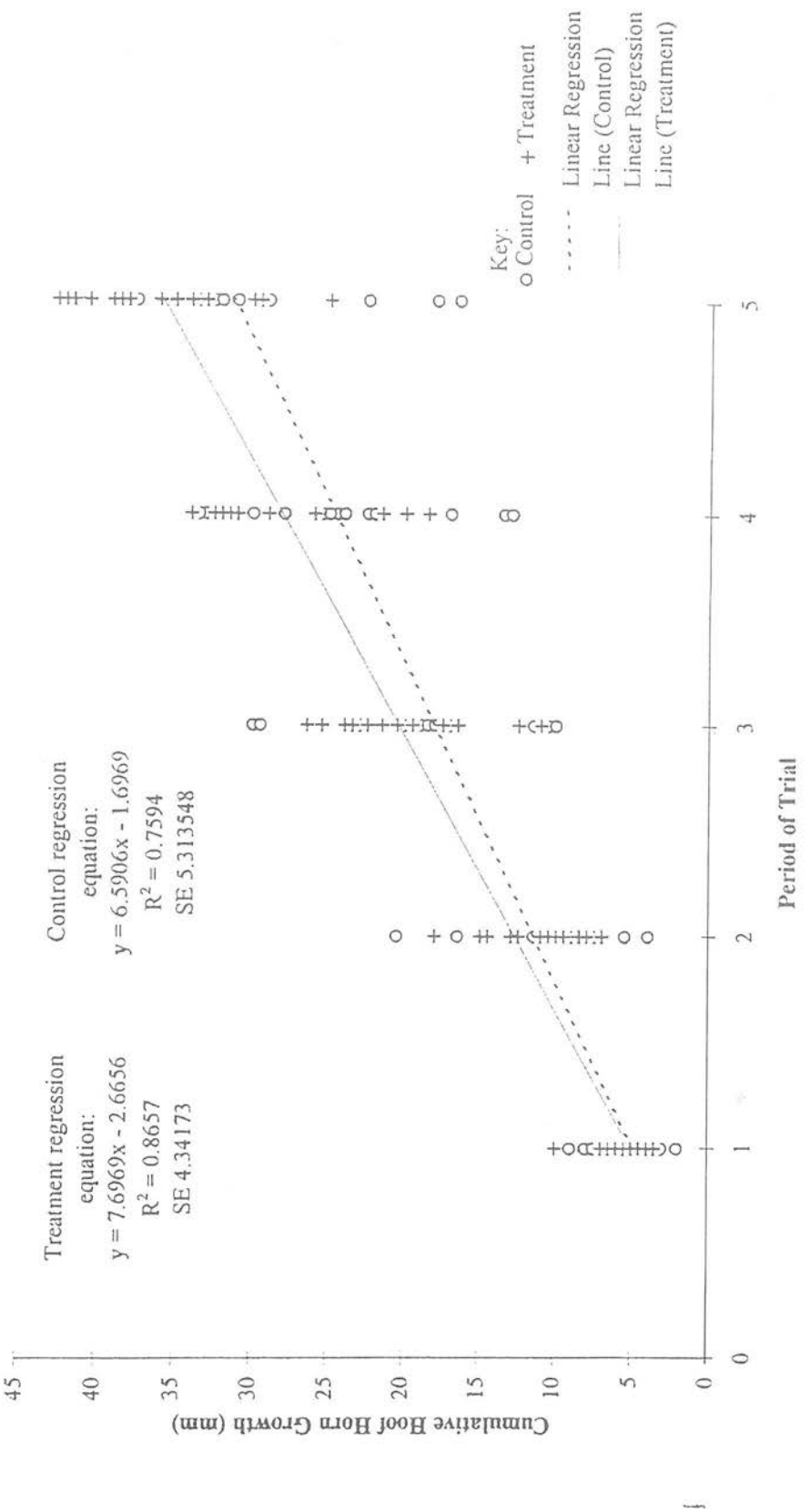
Figure 2.2: Cumulative mean hoof growth (all feet) by period of trial



Key:- Control
Treatment
* Significant difference at p<0.05
I Error bars = Standard Deviation

B
Start of Biotin Supplementation

Figure 2.3: Hoof growth rates at midline dead centre for treatment and control animals



Comparison of regression analysis showed that these two slopes were significantly different from one another and therefore biotin supplementation had produced a significantly higher growth rate of hoof horn in this trial ($p<0.02$).

The mean rate of hoof growth over the whole of the trial, by extrapolation from Figure 2.2 was:

0.164mm/day for non-supplemented ponies

and 0.189mm/day for biotin-supplemented ponies.

When expressed in percentage terms treatment animals had a 15% higher growth rate of hoof horn and 15% more hoof growth, at the midline dead centre, by the end of the trial period.

2.4.3 Differences in total growth of hoof horn between feet and between ponies

Table 2.4 (a-d) shows the analysis of variance (ANOVA) on total growth, by foot, and by individual pony, for treatment and control animals.

This showed that there was no difference between feet for all eight ponies (Table 2.4a and c) but that there was a difference between individuals for total growth ($p<0.05$) (Table 2.4 b and d). The two older animals (pony 4 and pony 8 which comprised pair 4) both had significantly reduced hoof growth compared with the rest by the end of the trial.

2.5 Discussion

2.5.1 Experimental design

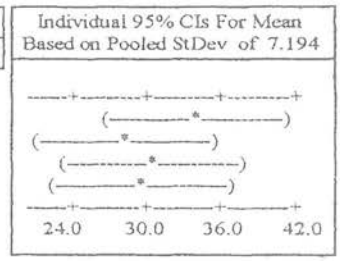
Restriction of experimental error in feeding trial design is desirable and is aided by controlling the extent of variability between animals. Experimental variability can be reduced by matching animals as nearly as possible (Roberts 1975). This has long been a principle in agricultural experiments. For example with dairy cows, matching by date of calving, parity, size, and previous milk yield are important and has been done for experiments leading to foot measurements in cattle (Reilly and Brooks 1990). For this experiment, because of the species used, and the nature of the measurements, it was more appropriate to match for breed, age, sex, size and weight. These are similar to the important factors to match for in beef trials (Roberts 1975). 'Growth' of horn is thus analogous to 'weight' of beef animal in such trials, and 'growth rate' of horn is analogous to 'growth rate' of the beast.

Table 2.4: Analyses of variance on total growth for individual feet and individual ponies

a) Control Animals - Analysis of Variance on Total Growth Between Feet

Source	DF	SS	MS	F	p
Foot	3	69.3	23.1	0.45	0.724
Error	12	621.1	51.8		
Total	15	690.4			

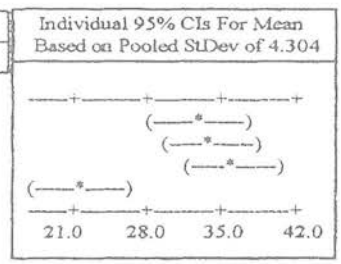
Foot	N	Mean	Standard Deviation
LF	4	34.125	5.186
RF	4	28.750	4.291
LH	4	30.500	9.600
RH	4	29.375	8.340



b) Control Animals - Analysis of Variance on Total Growth Between Ponies

Source	DF	SS	MS	F	p
Animal	3	468.2	156.1	8.43	0.003
Error	12	222.2	18.5		
Total	15	690.4			

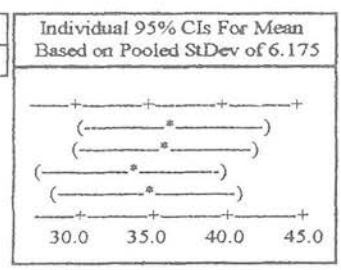
Pony	N	Mean	Standard Deviation
1	4	32.125	0.854
2	4	32.125	3.750
3	4	35.875	5.023
4	4	21.625	5.836



c) Treatment Animals - Analysis of Variance on Total Growth Between Feet

Source	DF	SS	MS	F	p
Foot	3	17.8	5.9	0.16	0.924
Error	12	457.6	38.1		
Total	15	475.4			

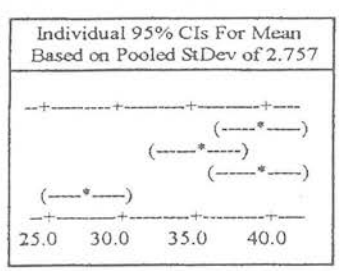
Foot	N	Mean	Standard Deviation
LF	4	36.625	5.344
RF	4	36.000	7.821
LH	4	33.875	3.521
RH	4	34.875	7.099



d) Treatment Animals - Analysis of Variance on Total Growth Between Ponies

Source	DF	SS	MS	F	p
Animal	3	384.17	128.06	16.85	0.000
Error	12	91.19	7.60		
Total	15	475.36			

Pony	N	Mean	Standard Deviation
5	4	39.500	2.041
6	4	35.250	0.957
7	4	39.250	4.213
8	4	27.375	2.750



In order to control for the vagaries of horse feeding in terms of variable inputs from hard feed, pasture or hay for example, a commercial cube was chosen as the basal diet and as the carrier for biotin supplementation. Although the rate of absorption of biotin into the bloodstream after feeding biotin in other forms is known (Lindner *et al* 1992), this assay was not performed in this experiment, as it was not in Buffa *et al* (1992), because of the prohibitively high cost of plasma biotin analysis. Initial hoof growth measurement between the potential treatment and control groups was carried out in only the last twenty six day period of basal feeding as assessment prior to then may have recorded the effects of nutritional and environmental factors affecting horn growth prior to purchase. The fact that there was no difference between the two groups meant that they could be confidently allocated without any carry over of covariate confounding factors. This acted as a form of internal control.

The biotin super-supplementation feeding experiment design was a "clinical feed trial" as defined by Elbers and Schukken (1995) i.e. it was a controlled comparative study of a treatment applied under natural, field or semi-field conditions. The trial conformed to this definition because the ponies were not kept in 'individual experimental stalls' in a controlled environment. They were being kept in as near to normal stabling conditions as possible with the exception that they were run as groups (because no individual stable space was available) and were housed on deep litter (because of lack of manpower for daily mucking out). The deep litter conditions were therefore not like normal stables but would be more like field conditions in that relatively less wear would take place on distal borders and this may have affected mechanics directly and indirectly via this possible effect on growth of horn. The ponies were also unshod and not hoof-trimmed during the trial. This was to minimise interference and possible farrier bias during the trial and was at the expense of those factors discussed above.

In this way, the 'experimental unit' under trial (Elbers and Schukken 1995) was the group of ponies i.e. treatment or control. The experimental unit is the smallest unit with a unique set of important characteristics (covariates like housing, feed, etc.) to which all the treatments in the trial can be assigned by randomisation (Elbers and Schukken 1995). The fact that there are only 2 experimental units (i.e. treatment and

control groups) decreases the number of degrees of freedom in the statistical analysis (Elbers and Schukken 1995). Thus it is not relevant to consider individual variation on an individual basis (as would with a trial set up in individual stabling) but as variation within the group. In this respect the group experimental approach used in this trial is not representative of the 'horse at large' but this was a constraint of the housing conditions available during the experimental period.

Nevertheless, according to Choraine (1993), the trial satisfies the requirements of a 'good clinical trial' apart from the fact that it was not a multi-centred trial and it was not blinded. According to Rossdale (1997), who considered the requirements for satisfactory clinical trials, the experimental work for this thesis satisfies the requirements of being randomised and controlled, but the work was not multi-centred or blinded. Blinding was desired but impossible because the author was the only source of manpower for all husbandry procedures. Thus any University feeding trial of this type can only really be a 'pilot study' by the definition used by Choraine (1993) because it only really helps define the variability of the parameters being studied since the numbers involved were constrained by finance. Nevertheless, the experimental design method of pairing by sex, weight and age and randomising one of each pair to treatment or control was done in order to enable a better homogeneity between groups and thus lead to less variability between groups. As a consequence, it increases the power of the study (Choraine 1993).

2.5.2 Hoof horn growth

The effect of biotin on hoof horn growth in this trial is in agreement with the findings of Bains (1985) and Buffa *et al* (1992). Can biotin be considered, then, a therapy? It can in so far as it has produced a change that will alter the 'renewal time' (Geyer and Schulze 1994) of the capsule for these ponies under these experimental conditions. From the probability of the differences in growth between control and treatment groups over time (See Figure 2.2) it is possible that the effect on the renewal time of the capsule would be more marked should supplementation have continued for longer. In addition, a small but significant difference in mean growth of 4.65mm in a treated foot compared with a non-treated foot over a period of 5 months, may just be sufficient to make a difference in new horn for nailing in to in an

overwintered animal, or to push away an old lesion which, as a result of the rather subtle mechanism of hoof wall growth and repair (Reilly *et al* 1996), does not rely on total capsular renewal to be replaced. In situations where total capsule regrowth is required, then biotin supplementation may help reduce 'renewal times' following hoof capsule avulsions or perhaps after major resections subsequent to laminitis or onychomycosis. This, however, assumes a similar response in resected horn which cannot be inferred from the results of this paper. Further work is required to investigate the effects of biotin as a therapy under these circumstances.

The results from this trial need to be interpreted according to the constraints of the trial and they should not be considered applicable to all ponies, or to horses, or even to animals that have different circumstances to the ones in the trial, eg other breed types, shod animals, or very young animals, until the work is repeated for these groups that have not represented in this trial.

The results from this trial therefore need verification either from repetition or from a multi-centred field trial approach. They also need to be verified in horses as opposed to ponies, and should not automatically extrapolated to horses, or to other species with hooves.

2.5.4 Hoof growth differences between feet and between individuals

There was no difference in growth between feet in this trial. This does not agree with the findings of Butler (1976) and Scott and Butler (1980). However these authors reported growth in foal groups as they aged from eight months old. The ponies in this trial were more mature. There is no clear evidence as to whether or not growth decreases with older age within the equine population, other than foals and yearlings appearing to have higher growth rates when a comparison between different workers' results is made from Table 1.1.

In this trial there was a difference in hoof growth between individuals by ANOVA (see Table 2.4), with the two older ponies showing a significantly decreased growth of horn compared with the others. Further work is required from trials designed to investigate the relationship between age of animal and response to biotin supplementation.

2.5.5 Hoof horn growth rates

The mean growth rates in this trial of 0.164mm/day (control) and 0.189mm/day (treated) over a winter period (October-February) are in agreement with a generalized figure, for mature horses, of 0.2mm/day (See Table 1.1) and with Glade and Saltzman (1985). This is lower than those recorded by Buffa *et al* (1992) but that investigation was conducted in South Africa, in a dry season and with horses that were exercised and shod.

Pollitt (1990) showed an inferred difference in hoof growth rate by dorso-palmar depth into the dorsal hoof wall, using an autoradiographic technique, with uptake and distal movement of the mark being slower in the inner hoof wall, nearer the SM, than in the outer hoof wall. Thus, measuring the rate of descent of a branded mark on the outside of the hoof capsule may only indicate part of what is happening within the hoof wall. Evidence for 'zonation' by dorso-palmar depth of the wall has been given by Reilly *et al* (1996), and is part of the work presented in Chapter 3.

How biotin exerts its effect is unclear. Buffa *et al* (1992) concluded that it influenced the amount or proportion of keratin molecules in hoof horn, resulting in increased growth rate. Kempson (1987), citing Marston (1948), implied that a response to biotin supplementation was brought about 'through modification of the division and maturation of proliferative cells'. To what extent biotin has effects during the cornification and/or keratinization processes cannot be concluded until measurement is undertaken at the cellular/molecular level. Otherwise, it can only be postulated that alleviation of a rate limiting step in the enzymatic functions of biotin may explain the responses in this trial.

This was a biotin super-supplementation experiment to a group of ponies that did not have overtly problematic feet. The lack of rationale for a response to super-supplementation in horses that should not theoretically be biotin deficient has been discussed by Buffa *et al* (1992). In that report the horses had 'poor hoof horn' by visual inspection; in this case the ponies did not. They were also not being worked, nor unduly stressed, and so a response to supplementation has been shown in a group of animals that should not have been biotin deficient and for which confounding factors had been minimized. Control ponies were receiving approximately 0.3mg of

biotin per day (Table 2.1) and the Treatment animals were receiving approximately 30mg of biotin per day. The treatment dose level is equivalent to approximately 60mg/day if extrapolated to a 500kg BW horse. Although the biotin requirements of horses have not been ascertained (Putnam 1986, cited in Lindner *et al* 1992), the control animals were receiving an acceptable level of biotin for maintenance according to Harris *et al* (1995) who recommend 0.1mg/kg of diet. Thus, the results from this trial can be regarded as a supraoptimal response (Cuddeford 1991) in ponies with normal feet. It is unknown whether a higher dose rate of biotin would effect bigger differences. Further work is required to be able to assess fully the response of the equid to supraoptimal supplementation with keratogenic factors. Future approaches to experimental work should, ideally, include double blind, placebo-controlled, cross-over designs.

2.6 Conclusions

The effect of dietary biotin supplementation, at a dose rate of 0.12mg/kgBW, on growth and growth rate of the hooves of eight match-paired ponies was investigated in a controlled feeding trial.

Treatment animals had a mean hoof growth at the midline dead centre of the hoof capsule of 35.34mm after 5 months of biotin supplementation. Control animals, with adequate biotin, achieved 30.69mm. This was a significant difference ($p<0.05$) by Student's t test.

Comparison of regression analysis also showed that biotin supplementation produced a significantly higher ($p<0.02$) growth rate of hoof horn in this trial.

Treatment animals had a 15% higher growth rate of hoof horn and 15% more hoof growth at the midline dead centre, after 5 months of biotin supplementation compared with Control ponies.

The treatment effect confirmed the findings of Buffa *et al* (1992) which were also from a controlled trial.

No differences were found between feet for growth of horn, but the older animals in the trial had significantly lower hoof growth ($p<0.05$) than the remaining ponies.

CHAPTER 3

Tubular and Intertubular Horn and Tubule Density

3.1 Introduction

The existence of tubular and intertubular horn in the hooves of cattle and horses has been known for some time (Nickel 1938, 1939; Wilkens 1964), although in the earlier literature the tubules were, confusingly, referred to as fibrils or 'horn filaments' (Fleming 1871). In this thesis the term fibril is reserved to describe the intracellular structural arrangement of keratin protein as seen in Figure 1.6. These tubules are assumed to be continuous, to be in the same plane as hoof wall growth and to run down the length of the wall from the coronary band to the bearing border of the hoof at the ground surface (Pollitt 1995). Hoof horn tubules are assumed to be continuous structures which are parallel with the wall (Greenough *et al* 1971) and this assumption is important with regard to counting of tubule density in this chapter and tubule area measurements presented in Chapter 4, as it allows for accurate perpendicular sectioning of tubules.

Perpendicular sectioning of tubules is important in order to produce accurate data for tubule density counting in this chapter and for absolute tubule area measurements given in Chapter 4. Any departure from a horizontal section would result in an overestimation of absolute tubular area measurements and produce false cross-sectional shapes (Hofstetter 1985). Also, the forces acting at the MDC are assumed to be symmetrical about this plane when the foot is balanced (Reilly *et al* 1996). This is important as Nickel (1938, 1939) and Pellmann *et al* (1993) have argued that tubule shape is in part, determined by the forces acting on them.

The hoof wall plays a major role in dampening the concussive forces of locomotion (Dyhre-Poulson *et al* 1994), and its arrangement as tubular and intertubular horn is believed to be important in stress transfer (Nickel 1938, 1939; Wilkens 1964).

Bertram and Gosline (1986) suggested that the arrangement of hoof horn tubules will have important consequences on the way in which forces are distributed within the equine hoof wall.

Nickel (1938, 1939) and Wilkens (1964) gave descriptions of the morphology of the tubules in the SM of the equine and bovine hoof walls and described the

differences in tubule size, orientation of component cells, and fibrillar arrangement within tubular cells for different parts of the wall. However, neither author quantified the distribution of these tubules throughout the wall. Leach (1980) and Stump (1967) gave further unquantified reports of the distribution of tubules within the equine hoof wall. Differing mean tubule densities for horses of approximately 30/mm^2 (Leach and Zoerb 1983) and 8-14/mm^2 (Bucher 1987) are quoted. However, the methods on which these figures are based are not reported.

Limited data are also available for other species: Geyer and Tagwerker (1986) reported a figure of 80-100 tubules/mm^2 of horn for the dorsal hoof wall in pigs. Kempson *et al* (1989) reported a similar mean figure for TD of 99/mm^2. Neither of these studies presented the methods nor basic descriptive statistics for calculating the tubule densities reported. For cattle, an average of 80 tubules/mm^2 of horn for the dorsal hoof wall was quoted by Vermunt and Greenough (1995). For sheep, Rosskopf and Geyer (1987) reported 99 tubules/mm^2. No objective reports of the distribution of the tubules within the hoof walls of these species have been given either. Tubule densities for hoof tissues from different species are given in Table 3.1

Table 3.1 - Hoof Wall Tubule Density for Different Species

Animal	Tubule Density (Tubules/mm^2)	Method	Authors and Year
Horses	30	Light microscope	Leach (1980)
Horses	8-14	Light microscope	Bucher (1987)
Cattle	80	Unknown	Vermunt and Greenough (1995)
Sheep	99	Light microscope	Rosskopf and Geyer (1987)
Pigs	80-100	Light microscope	Geyer and Tagwerker (1986)
Pigs	99	Electron microscope	Kempson *et al* (1989)

It is believed that 'horn quality' is determined to a large degree by the number of tubules per unit area in the cow (Politiek *et al* 1986) and in the pig (Geyer and Tagwerker 1986). 'Hardness' of horn is believed to be directly related to the number of tubules per unit area in cows (Gunther *et al* 1983) and in pigs (Geyer and Tagwerker 1986). However, as discussed in Chapter 1, there is a lack of objective definition of 'horn quality' (Reilly 1995) and few established relationships reported between mechanical properties and structural features of the hoof.

It was described in Chapter 1 that there is confusion as to the role of the tubular and intertubular formations within the hoof wall and there has been imprecise quantification of this feature. Inter-relationships with mechanical measurements for morphological features found for the equine hoof are reported in Chapter 6. However, in order to be able to attempt an investigation of inter-relationships, the basic data for tubule density needed to be obtained.

3.2 Aims

The aims of this Chapter are to:

(i) give a method by which tubule density may be obtained in hoof horn samples from the SM of the hoof wall of normal ponies. This provides an objective tool for the investigation of the properties of hoof horn.

(ii) use this method to determine whether there is any alteration of this parameter by biotin supplementation.

3.3 Materials and Methods

3.3.1 Determination of Sample Site, Hoof Dissection and Sample Preparation

At the end of the 5 month feeding experiment the ponies were humanely killed at an abattoir. Distal limbs were disarticulated at the fetlock after labelling. The hooves were wrapped in a double layer of cling film to prevent dessication, wrapped in waterproof bags and transported to the laboratory on ice.

The animals were killed because full depth SM samples were required for subsequent quantitative testing and it was considered inhumane, and therefore against veterinary ethics, to carry out the alternative method of obtaining full depth SM samples, which was full depth biopsy. The duration of the feeding experiment (5 months) did not allow time for full capsular renewal and so hoof clippings were not available. In any case the use of the hoof clipping as a source of sample material is flawed as the properties it displays are confounded by the effects of wear (Vermunt and Greenough 1995), mechanical damage (Josseck *et al* 1995), environmental degradation by urine and faeces (Albarano 1993, Kung 1991) and possibly farriery although this would not have applied in this case as the animals did not have farriery procedures during the course of the trial. In addition, these factors can not be controlled between pairs. For example, differential growth and wear patterns could

mean that material of dissimilar 'age' could be sampled in a clipping unless a measurement of wear was to take place. The objective information obtained from the measurement of horn growth between pairs and given in Chapter 2 meant that like samples could be taken for subsequent analysis once the site of sampling within the precise region of new horn growth had been determined. The way this was achieved is given in section 3.3.1.1 below and thus, even though it was known from results in Chapter 2, that treatment and control hoof horn at the MDC grew at different rates during the trial, like sampling between pairs was achieved.

3.3.1.1 Determination of New Horn Growth

The initial brand marks were placed at variable distances below the periople as described in Chapter 2. The absolute distance to the RH_L was measured after branding to give an initial reference point, RH_LG_0 in Table 2.3.

At the end of the period of the trial, the brand mark had descended to point RH_LG_5 (see final column Table 2.3). Therefore, RH_LG_5-RH_LG_0 is the total growth of hoof horn as measured by the descent of the brand and gives the equivalent growth of new horn (Gn in Figure 2.4) when measured from the CB.

Thus, in order to ensure that the sampling of horn for histomorphometry was made within new horn (i.e. that horn which had been genererated under the experimental conditions of the trial), the following calculation was made to identify the region of new horn growth, which also allowed for any difference in growth between pairs, due to the treatment effect which was described in Chapter 2.

For example, from Table 2.3.

For Treatment Pony 1 (Pony 5):

RH_LG_0 29 mm, RH_LG_5 = 68mm, RH_LG_5- RH_LG_0 = Gn = 39mm

for Control Pony 1 (Pony 1):

RH_LG_0 19 mm, RH_LG_5 = 51mm, RH_LG_5- RH_LG_0 = Gn = 32mm

Thus, in order to establish the distal limit of new horn growth (DLNHG), the distance given by RH_LG_5- RH_LG_0 was then measured from the anterior MDC coronary band, $\overline{CB\ DLNHG_t}$ for treatment animals within a pair and $\overline{CB\ DLNHG_c}$ for control animals within a pair, as shown in Figure 3.1.

In order to take account of the difference in growth between pairs due to treatment effect and to ensure that sampling took place at the same gross anatomical site between pairs, the lowest absolute value for DLNHG, whether for control or treatment animal, for each pair, was used to determine the reference point for sampling, RPS, within each pair as shown in Figure 3.2.

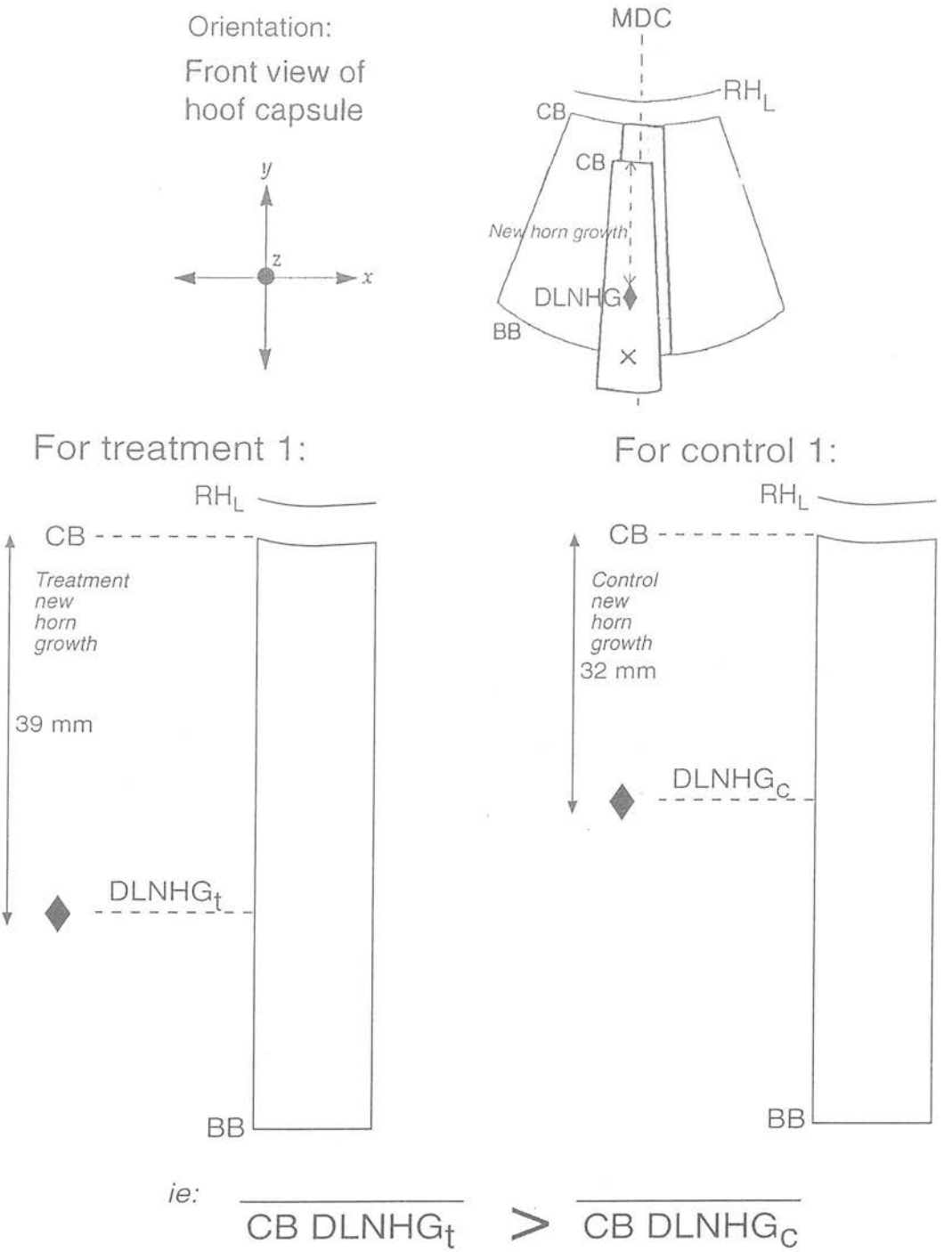
Once the RPS had been established between pairs the site of sampling of new and like material for testing between pairs was known. Thus, material was provided for cutting of sample blocks as follows:

Four sample blocks were required in total. Three, 2mm deep, samples were required for mechanical testing (described in Chapter 5) and one, 2mm deep, sample was required for histomorphometry (described in Chapter 4). Thus, allowing for a 0.5mm loss of material due to the destructive action of the circular saw blade used to prepare samples from the blocks, the distance to the position of a CUT POINT, with respect to the RPS, was calculated to be 10mm proximal to it, to allow like sampling within new horn. The 10mm distance between CUT POINT and RPS allowed for 4 x 2mm blocks with 0.5mm loss between them as shown in Figure 3.3.

A summary flow diagram for sampling procedures is given in Figure 3.4.

Since no difference had been found between individual feet in the same animal in terms of growth or growth rate response as described in Chapter 2, the left fore (LF) foot from each pair of animals ('treatment' and 'control' pair) was used to give samples for further quantitative tests. Since no difference had been found between feet they could not be judged to be independent of each other. Any biotin effect, or lack of effect, could reasonably be expected to occur with the same probability in each foot equally and to have used samples from each foot from each individual animal would have artifically inflated the number of contributing values (n) without true replication. This would simply have created more samples and would have been statistically unsound as this would have created a 'pooling fallacy' (Machlis *et al* 1985). The use of the LF only to provide material also follows the convention of previous workers (Barr *et al* 1995, Buffa *et al* 1992 and Webb, Penny and Johnston, 1984).

Figure 3.1: Determining the Distal Limit of New Horn Growth at the Midline Dead Centre for individuals in a pair. (not to scale)

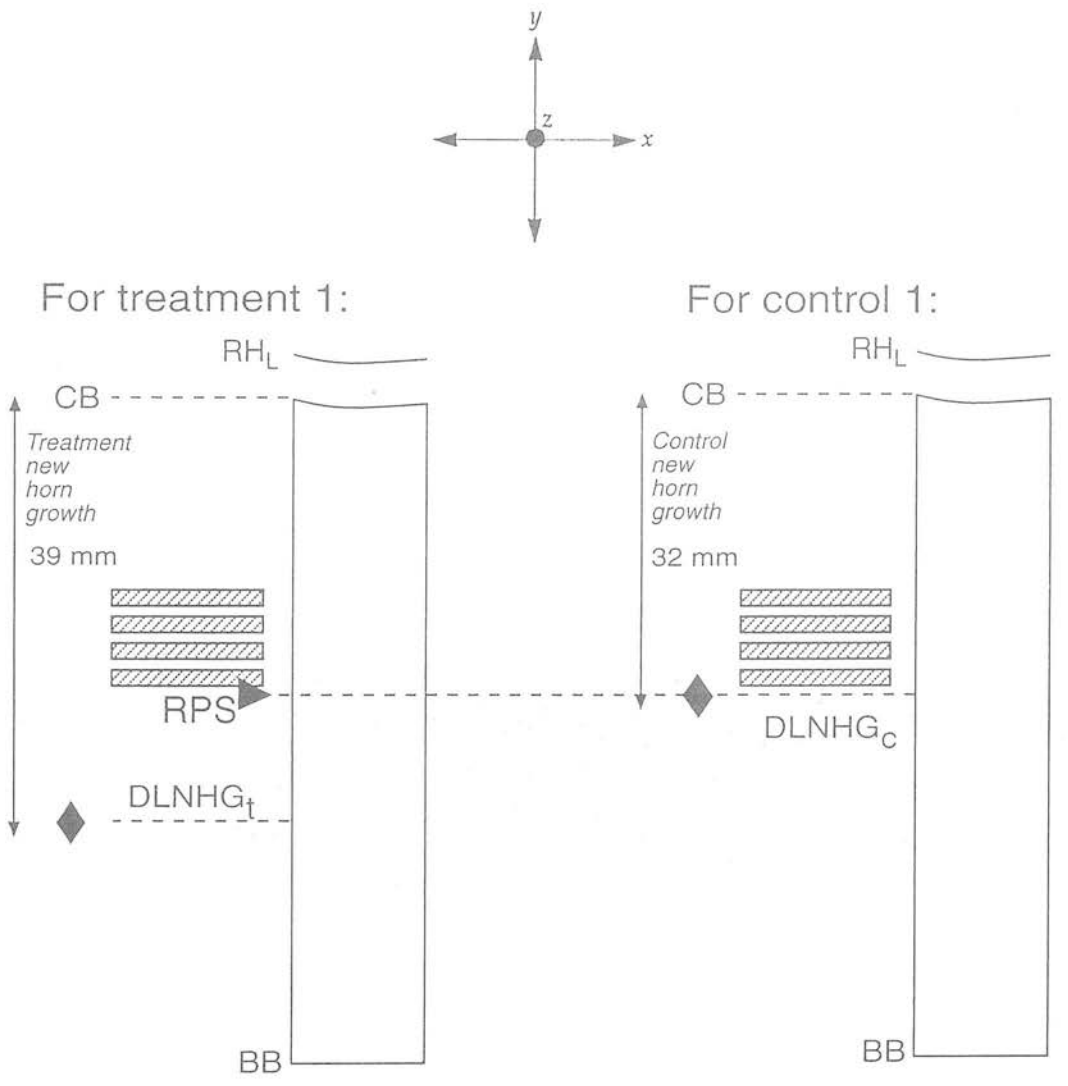


ie: $\overline{\text{CB DLNHG}_t} > \overline{\text{CB DLNHG}_c}$

Key

CB: Coronary band
BB: Bearing border
RH$_L$: Reference hair line
X: Brand mark on hoof
♦ DLNHG$_t$: Distal limit of new horn growth for treatment animal
♦ DLNHG$_c$: Distal limit of new horn growth for control animal

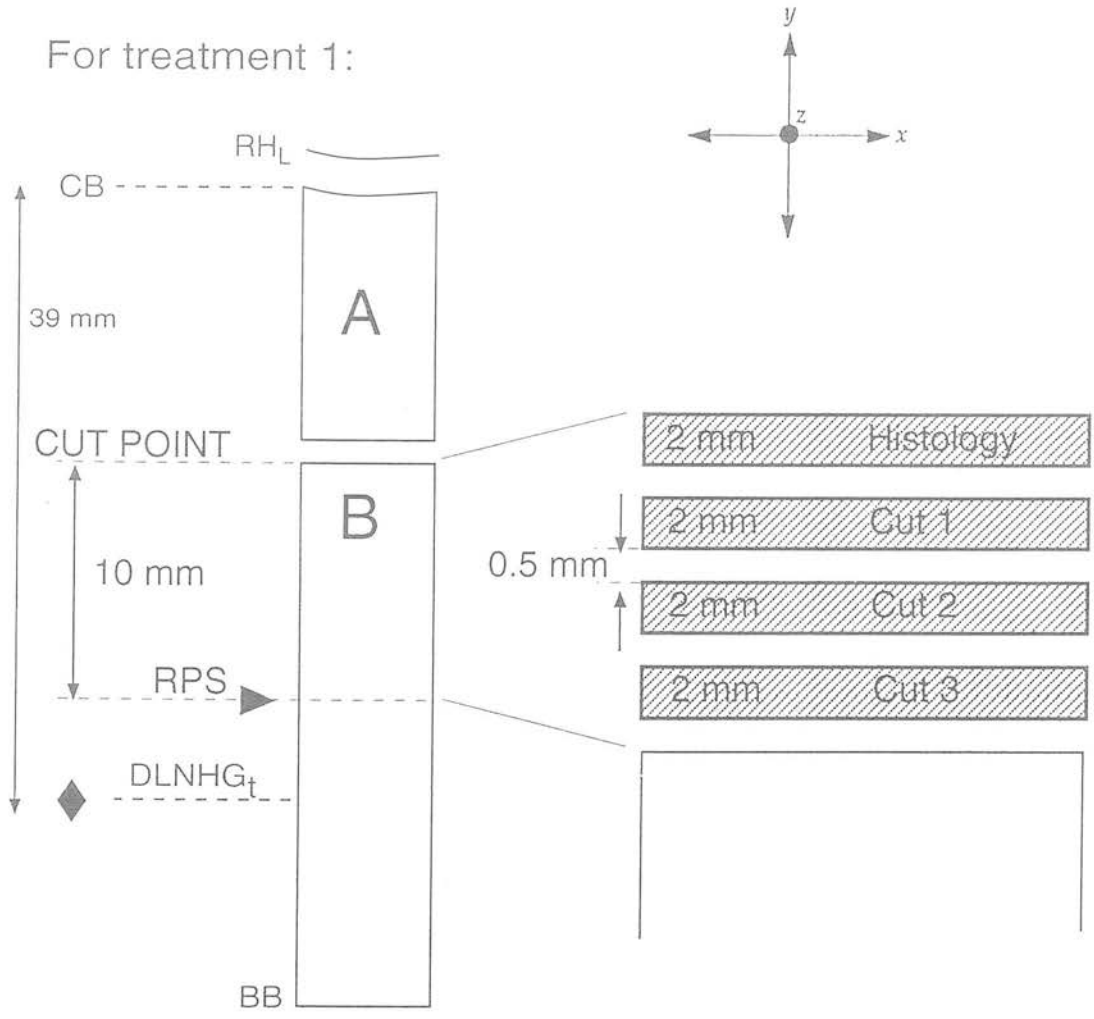
Figure 3.2: Determining the Reference Point for Sampling at the Midline Dead Centre for individuals in a pair (not to scale)



Key

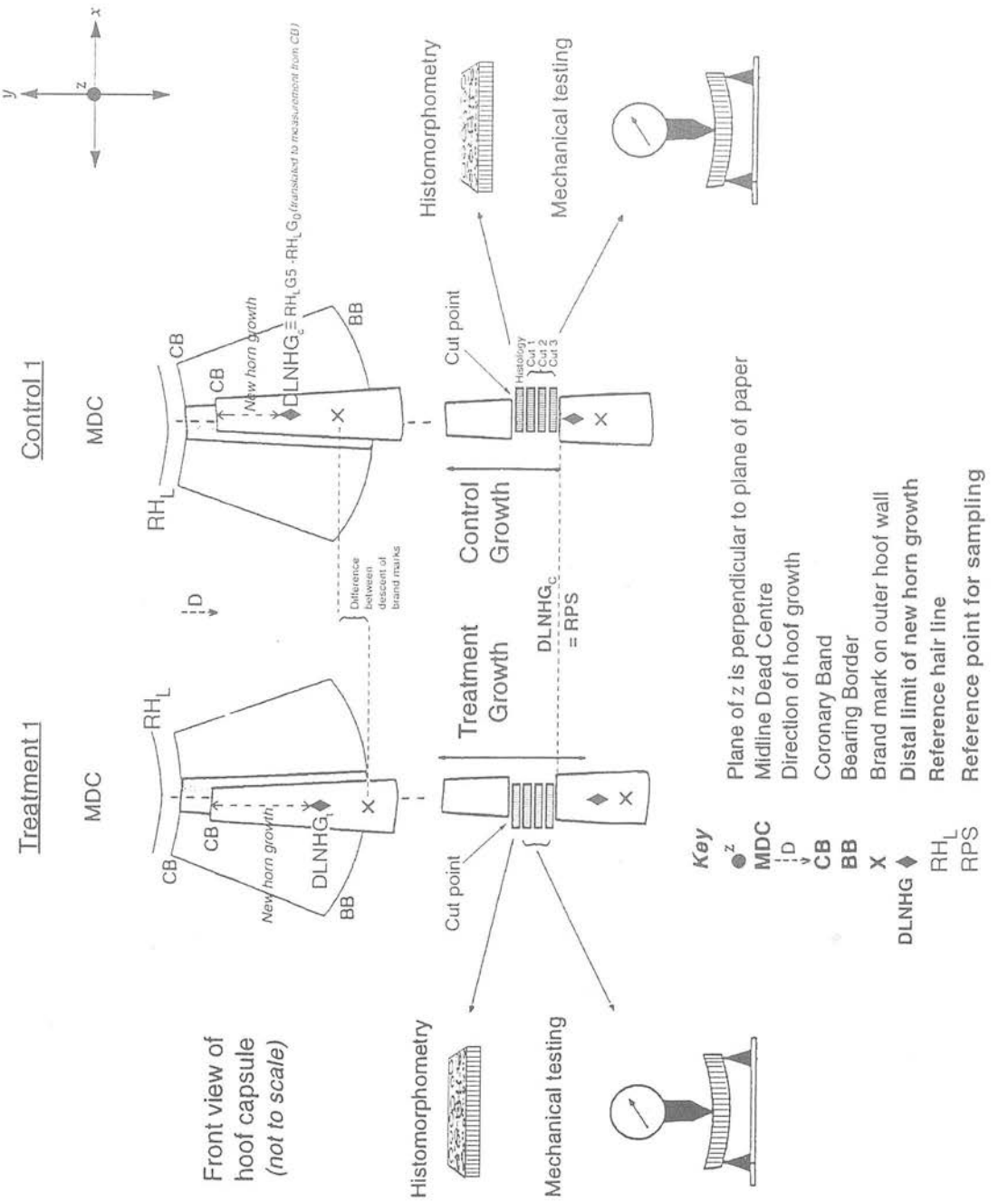
CB: Coronary band
RH$_L$: Reference hair line
BB: Bearing border
X: Brand mark on hoof
♦ DLNHG$_C$: Distal limit of new horn growth for control animal
RPS: Reference point for sampling
▨ Sample blocks

Figure 3.3: Determining the CUT POINT at the Midline Dead Centre for individuals in a pair. (not to scale)



Key
CB: Coronary band
BB: Bearing border
DLNHG$_t$: Distal limit of new horn growth for teatment animal
RPS: Reference point for sampling
RH$_L$: Reference hair line
A: Proximal block subsequently stored in formal saline
B: Block for sample cutting
▨ Sample blocks

Figure 3.4: Flow diagram to show sampling of hoof horn for quantitative tests



3.3.2 Histology and Determination of Tubule Density

Individual samples for histology were held in OCT embedding matrix (Cell Path Ltd) on microtome chucks. Horizontal sections, in the z plane (Figure 3.4), of 10μ thickness were cut from each sample using a Bright™ cryostat model OTF/AS fitted with a D profile steel microtome blade at a cutting angle of 10°. The specimen was orientated such that sections were cut from the direction of the SI tangentially through towards the SE (see Figure 3.5). That is, the specimen blocks were mounted on chucks at an angle to the microtome blade, in order to allow the blade to move gradually from soft SI to much harder SE. The sections were initially cut at 20μ to produce a flat cutting surface devoid of any saw blade marks and then subsequent sections were cut at 10μ thickness. They were stained in haematoxylin and eosin (H&E), dehydrated, and mounted in 'DPX' mounting medium (Merck Ltd) with a cover-slip. The protocol for H&E staining is given below:

H&E Staining Protocol for TD Counting:(From Geyer, H. personal communication)

Wash cut sections by floating in cold water bath

4 minutes in Ehrlich's haematoxylin

Rinse in water

Dip in acid alcohol

Water for 5 minutes

Eosin for 30 seconds

Dehydrate through a series of alcohols (70%, 90%, 100%)

Mount in DPX

When the prepared sections were viewed through a light microscope at low power (x2.5 objective) the field drawn in Figure 3.6 was seen. Photographs were taken of each section at a given magnification. A corresponding photograph was taken of a 1mm calibration graticule, at the same magnification, to act as a scale. In this way, a scaled photographic print of the microscope image was obtained. This was then magnified, as was the scale, to A4 proportions for ease of working. An example of the grid superimposed on a sample from Pony 6 (Treatment Animal 2) is given in Figure 3.7.

Consistent alignment of the grid, between sample prints in terms of an overlying 'grid' that was to be superimposed for TD counting, was achieved by

Figure 3.5: Cutting midline dead centre hoof horn sections for histology on the cryostat

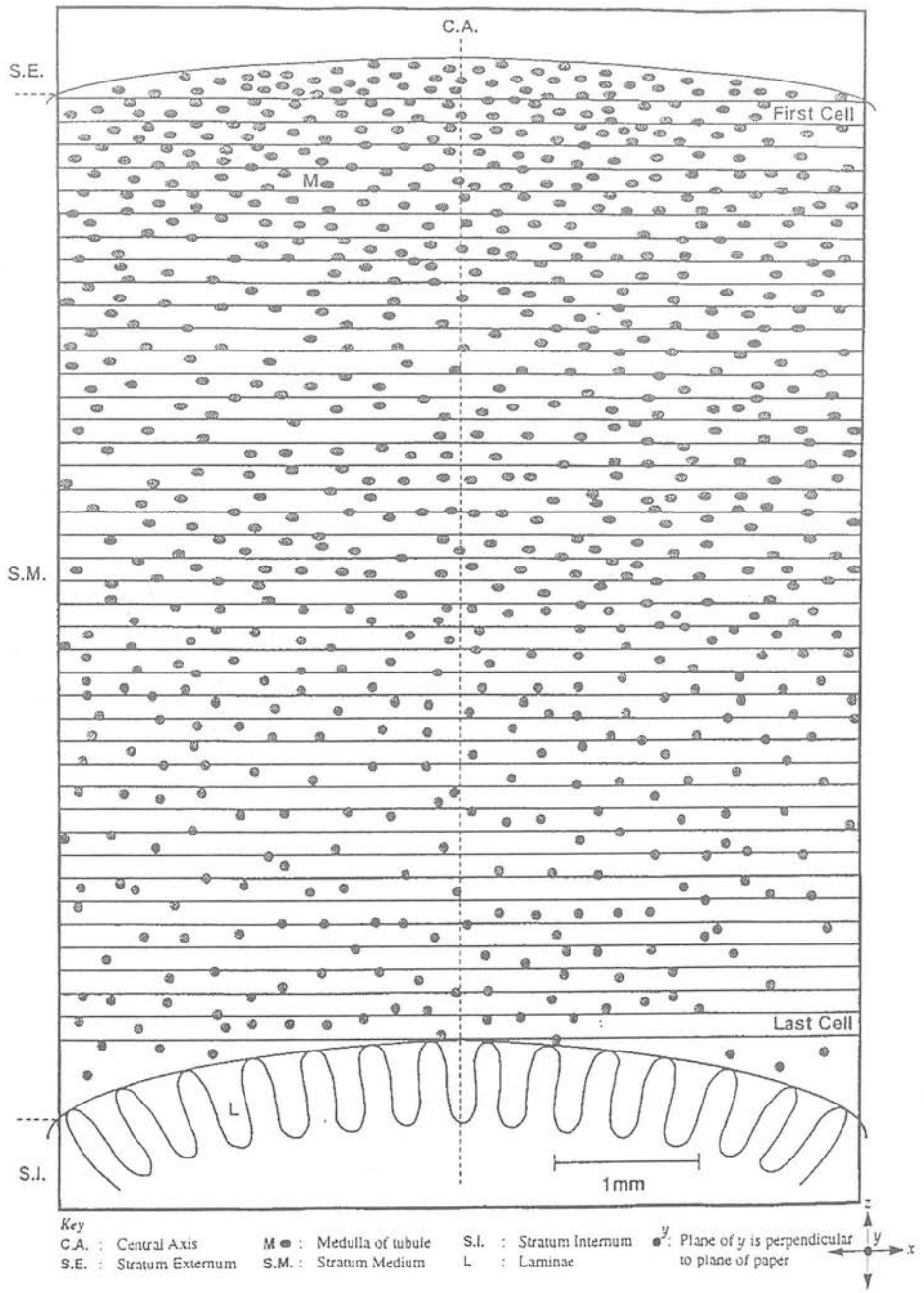
Key

SI = *stratum internum*

SE = *stratum externum*

= direction of cut

Figure 3.6: Defining boundaries for tubule density counting



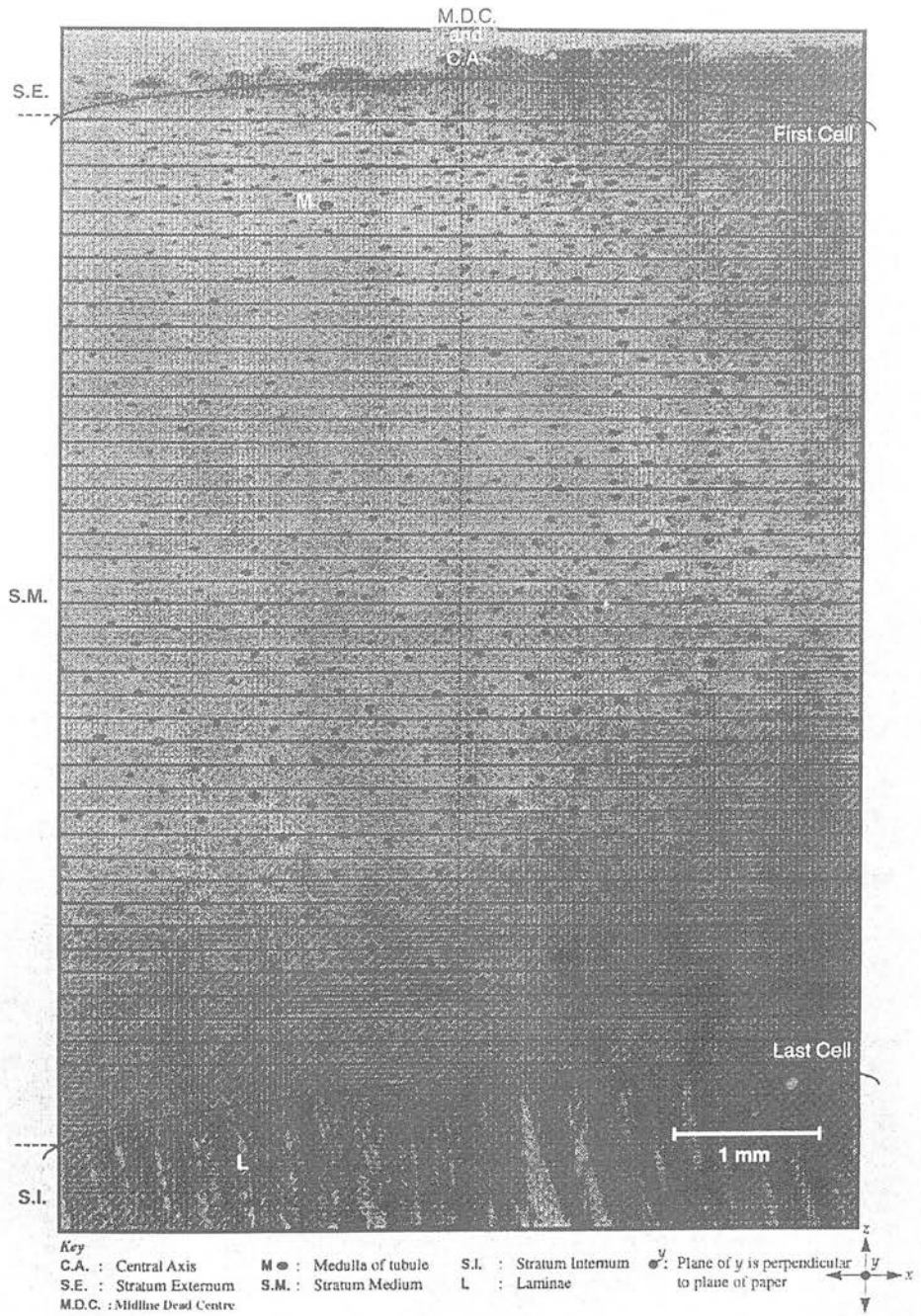
defining a 'central axis' (CA) for each section which corresponded to the MDC (see Figures 3.6 and 3.7).

The outer surface of the hoof capsule, or *stratum externum* (SE in Figure 1.6 and Figure 3.7), has a curved exterior in the x plane. The inner surface, or *stratum internum* (SI in Figure 1.6 and Figures 3.6 and 3.7), is made up of a 'fan' of radially oriented laminae (L in Figure 1.6 and Figures 3.6 and 3.7). The SI has a different curvature to the SE and the forward projections of the laminae do not always form a smooth line. The CA was given by a line which was in the centre of the histological section and which ran from the curvature of the SI to meet the SE at a right angle (--CA-- in Figures 3.6 and 3.7). The CA was consistent with the MDC (Figure 3.7) since the CA represented the centre of a section that had been cut from the hoof wall symmetrically about the MDC.

Since a concentration of stain was taken up by tubule marrows this defined the location of any given tubule within the SM of the hoof wall ($M_{\bullet}$ in Figures 3.6 and 3.7). A grid of parallel horizontal lines, at 0.5mm intervals, was then superimposed on the A4 photographic print such that its lines were perpendicular to the CA. The area between grid lines determined the boundary for counting tubules in the z plane. The width of the photographic field determined the boundary for counting between the grid lines in the x plane. The area thus bounded between grid lines was referred to as a 'cell'. Alignment of the grid in the z plane was such that the first cell for counting within was the one in which the full width of field was covered by SM ('First cell' in Figures 3.6 and 3.7). The final cell that contributed to the count in the z plane was that which gave a full width of field without being interrupted by any part of a lamina (L) from the SI (See 'Last Cell' in Figures 3.6 and 3.7).

In this way tubule counting, in relation to the area between the grid lines, by depth into the hoof wall, was facilitated. The convention employed for counting within a cell was to ignore stained marrows that touched the upper and right-hand boundaries, but to count all other marrows within the cell including those that touched the lower and left-hand boundaries. If a marrow at the top of the cell 'touched' the

Figure 3.7: Histological sample to show division of the stratum medium of the hoof wall for tubule counting (not to scale)



cell boundary but was otherwise wholly enclosed within the cell it was counted. This convention is summarised in Figure 3.8. This method gave the number of tubules per cell, which was termed the 'initial tubule density' (ITD).

Thus, the initial tubule density (ITD) was the number of tubule marrows that were counted per unit area of cell within the grid.

Tubule density (TD) was the ITD converted to give the equivalent number of tubule marrows per unit area of hoof horn tissue proper (i.e. in reality). Knowing the size of the cell (from the product of the scaled cell dimensions), the TD mm^{-2} of horn was calculated as follows:

Eg: For cells in Figure 3.7 (pony 6): (Calculations given for original A4 size).

Grid cell dimensions = 17.0 cm x 0.5 cm

Scale bar = 3.1 cm : 1 mm

Thus, scaled horn dimensions within cell in reality = 17 / 3.1 x 0.5 / 3.1

$= \underline{0.88\ mm^{-2}}$

(= conversion factor, CF)

To convert an ITD of, say, 25 tubules per cell to a TD:

TD = ITD / CF

= 25 / 0.88

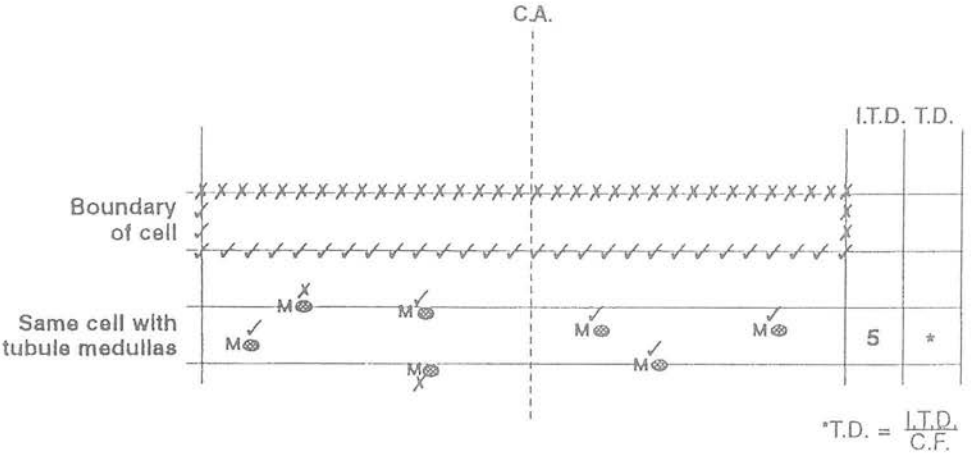
$= \underline{28.41\ mm^{-2}}$

Different animals had different depths of hoof wall in the z plane (i.e. different depths of SM). Thus, with the arbitrary grid chosen, each animal had a different number of cells per wall depth as seen in Table 3.2. To normalise for these differences in hoof wall depth between individuals, the tubule density data was expressed by percentage hoof wall depth (%HWD). The %HWD represented by each cell in each case was calculated by:

$$\%\ \text{HWD per cell} = \frac{100\%}{\text{Total number of cells from 'First cell' to 'Last cell'}}$$

This meant that for each animal the fixed cell dimensions represented a different percentage of the wall depth for that animal.

Figure 3.8: Tubule counting method



Key

C.A. : Central Axis
✓ : Counted
✗ : Not counted
M⊗ : H+E stained tubule medulla
I.T.D. : Initial Tubule Density
C.F. : Conversion Factor
T.D. : Tubule Density

The values obtained for TD and for %HWD were then expressed as integers as shown in Table 3.2.

Data analysis, graphical and tabular presentations were completed using 'Minitab' and 'Statview' statistical packages on an Apple Macintosh personal computer.

3.4 Results

3.4.1 Tubule Density

The results for tubule density by depth into the SM of the LF hoof wall for each of the 8 ponies is given in Table 3.2.

A frequency distribution histogram of the tubule density data set from Table 3.2 is given in Figure 3.9 together with the basic descriptive statistics for this data set.

From Figure 3.9 it can be seen that tubule density has a non-normal distribution and is skewed to the right. The mean and median for the data set are not the same.

Figure 3.10 shows a square root transformation of Figure 3.9 and this gives a normal distribution. A set of basic descriptive statistics for the square root transformed data set is given and the mean and median are seen to be the same.

The results for tubule density by %HWD from Table 3.2 are presented as a scattergram in Figure 3.11.

The results show a distinct pattern of tubule distribution within the midline dead centre SM of the hoof horn from these ponies. Figure 3.11 shows a stepped pattern in the distribution of TD by HWD with two areas of decreasing density interspersed by two areas of relatively unchanging tubule density.

Using the basic descriptive statistics given in Figure 3.10, the transformed tubule density data set can be divided up by conventional use of $\pm$ 1 and 2 standard deviations about the mean to give divisions at 1.75, 2.89, 4.03, 5.17 and 6.31 square root (sq rt) tubules/mm^2. These five divisions correspond to TDs on the y axis of Figure 3.11 of 3.0, 8.35, 16.24, 26.7 and 39.8 tubules/mm^2 respectively. In order to extrapolate where the 4 zones between these 5 divisions would correspond to on the x

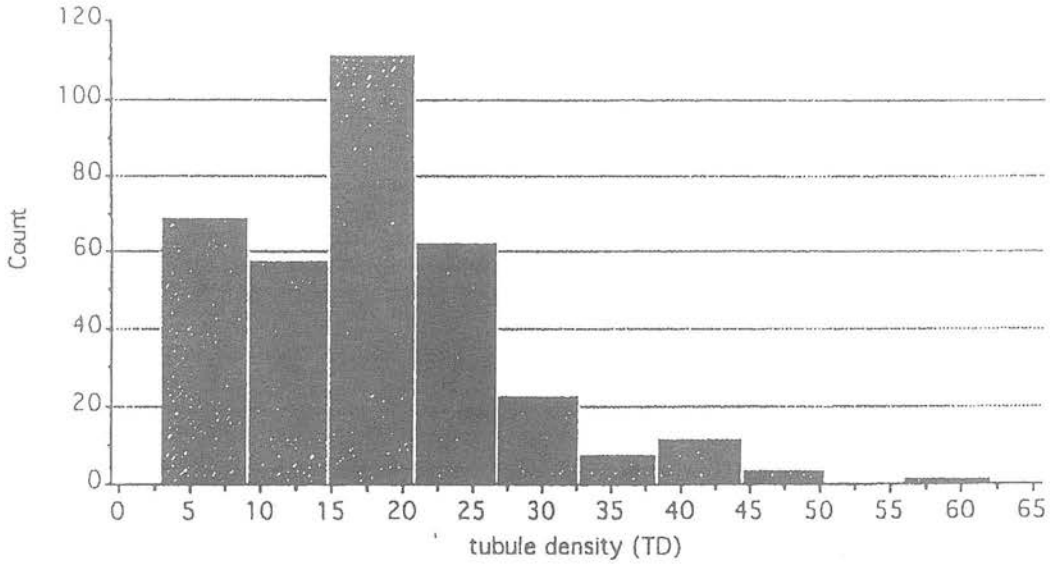
Table 3.2: Data set for Tubule Density (TD) and Percentage Hoof Wall Depth (%HWD) for the 8 Trial Ponies

'First cell' level ('outer' S.M)

'Last cell' level ('inner' S.M)

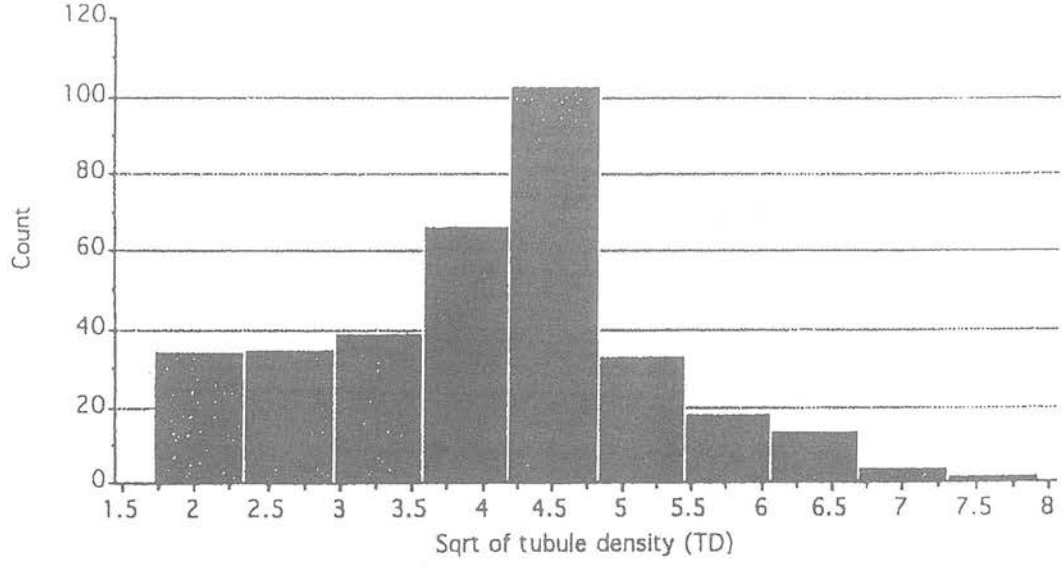
Control Animals								Treatment Animals							
Pony 1		Pony 2		Pony 3		Pony 4		Pony 5		Pony 6		Pony 7		Pony 8	
TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD
61	3	27	2	49	2	40	2	39	2	47	2	40	3	40	3
48	5	24	5	43	4	35	5	38	4	40	4	40	5	35	6
40	8	19	7	40	6	40	7	30	6	32	6	39	8	31	10
38	11	20	10	30	8	28	9	31	8	29	8	34	11	30	13
32	14	20	12	31	10	26	12	24	10	29	10	34	14	28	16
22	16	19	14	30	12	26	14	23	12	29	12	34	16	31	19
23	19	18	17	30	13	22	16	16	13	26	14	20	19	23	22
22	22	23	19	31	15	23	19	19	15	23	16	26	22	26	26
22	24	24	21	28	17	23	21	15	17	23	18	22	24	26	29
22	27	15	24	24	19	23	23	16	19	23	20	20	27	24	32
23	30	15	26	27	21	19	26	16	21	21	22	23	30	19	35
19	32	16	29	23	23	23	28	16	23	20	24	20	32	23	38
20	35	18	31	20	25	19	30	18	25	22	26	24	35	24	42
18	38	15	33	24	27	20	33	19	27	18	28	20	38	18	45
14	40	18	36	26	29	16	35	15	29	17	30	23	40	22	48
15	43	15	38	20	31	24	37	19	31	20	32	24	43	23	51
16	46	18	40	32	33	23	40	18	33	18	34	26	46	20	54
16	49	16	43	28	35	24	42	19	35	17	36	26	49	19	58
14	51	14	45	22	36	20	44	16	36	17	38	22	51	16	61
14	54	16	48	26	38	24	46	14	38	18	40	18	54	18	64
15	57	18	50	20	40	20	49	18	40	17	42	19	57	18	67
14	59	14	52	20	42	16	51	15	42	20	44	20	59	16	70
13	62	16	55	23	44	23	53	16	44	15	46	19	62	19	74
13	65	19	57	23	46	20	56	22	46	18	48	18	65	12	77
13	68	16	60	23	48	18	58	15	48	16	50	16	68	12	80
5	70	18	62	18	50	19	60	19	50	18	52	14	70	12	83
9	73	14	64	26	52	15	63	22	52	17	54	5	73	8	86
9	76	11	67	20	54	15	65	20	54	16	56	12	76	7	90
5	78	12	69	26	56	15	67	14	56	15	58	12	78	7	93
6	81	12	71	23	58	12	70	18	58	17	60	4	81	8	96
8	84	7	74	23	60	7	72	14	60	13	62	12	84	4	99
6	86	4	76	18	61	12	74	19	61	17	64	7	86		
5	89	5	79	22	63	5	77	15	63	13	66	7	89		
5	92	11	81	15	65	12	79	15	65	13	68	7	92		
4	94	4	83	16	67	9	81	16	67	11	70	5	94		
4	97	9	86	11	69	8	84	9	69	7	72	12	97		
4	100	4	88	13	71	5	86	14	71	9	74	7	100		
		8	90	8	73	5	88	9	73	11	76				
		3	93	4	75	12	91	8	75	11	78				
		7	95	9	77	8	93	9	77	9	80				
		4	98	4	79	8	95	8	79	7	82				
		9	100	7	81	8	98	8	81	7	84				
				3	83	7	100	5	83	10	86				
				7	84			8	84	10	88				
				8	86			4	86	9	90				
				3	88			8	88	10	92				
				4	90			4	90	12	94				
				5	92			9	92	10	96				
				9	94			5	94	6	98				
				5	96			9	96	6	100				
				4	98			4	98						
				3	100			7	100						

Figure 3.9: Frequency distribution of tubule density data



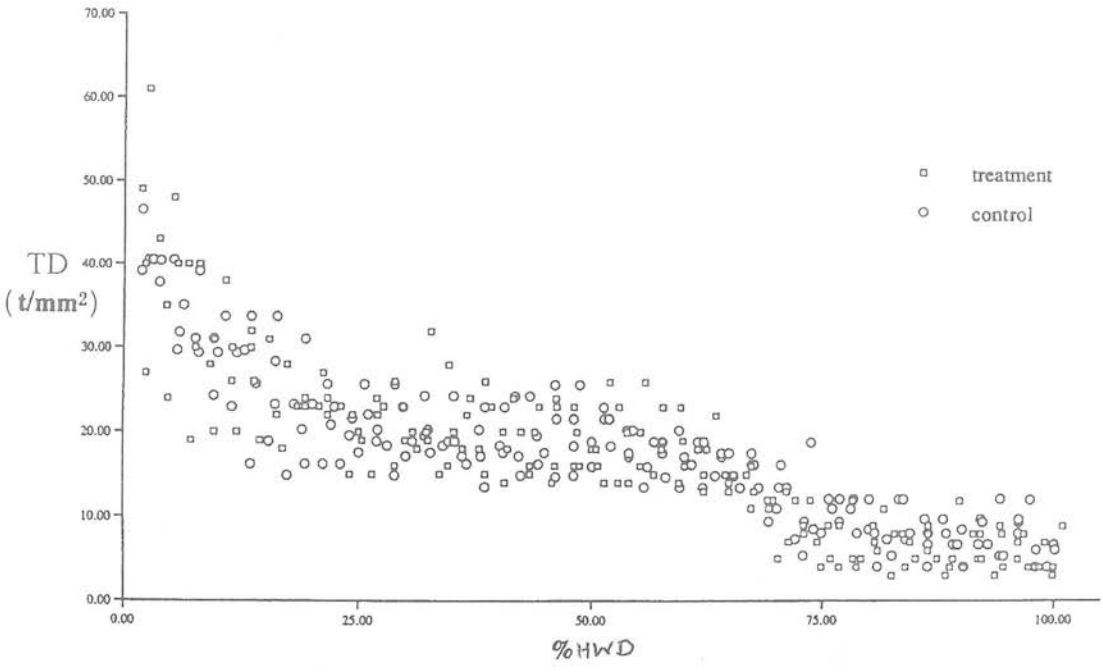
Count	Mean	Median	Std. Dev	Kurtosis
344.00	17.53	17.00	9.43	1.44

Figure 3.10: Frequency distribution of square root transformed tubule density data



Count	Mean	Median	Std. Dev	Kurtosis
344.00	4.03	4.03	1.14	-0.15

Figure 3.11: Scattergram of tubule density data (TD) by percentage hoof wall depth (%HWD) for all ponies



axis of Figure 3.11 (i.e. %HWD) a simple regression equation was used to describe the relationship between sq rt TD and %HWD. The equation is as follows:

%HWD = 141 - 2.3 sq rt TD

By substituting the above values of sq rt TD at standard deviation divisions for x in the above equation, values for y (%HWD) were given at: 76.55%, 51.13% and 25.71%, which splits the SM into 4 zones. These values occur at approximately 25% intervals and appear to correspond to where the approximate inflections of a curvilinear 'line of best fit' would occur in Figure 3.12.

3.4.2 Effect of biotin supplementation on tubule density

At first glance the scatter plot of TD for treatment and control groups would appear to show no differences from one another (see Figure 3.11).

However, mean values for:

Control TD @ 17.33 ± 10.06 (non-transformed) and 15.84 ± 1.46 (transformed)

Treatment TD @ 17.74 ± 8.76 (non-transformed) and 16.65 ± 1.02 (transformed)

which showed no significant differences by Mann-Whitney U tests or by t-test may have masked a real difference between the zonal distribution of TD between treatment and control groups.

Regression lines were added to the zonal tubule densities for zone delimited treatment and control group data in Figure 3.12.

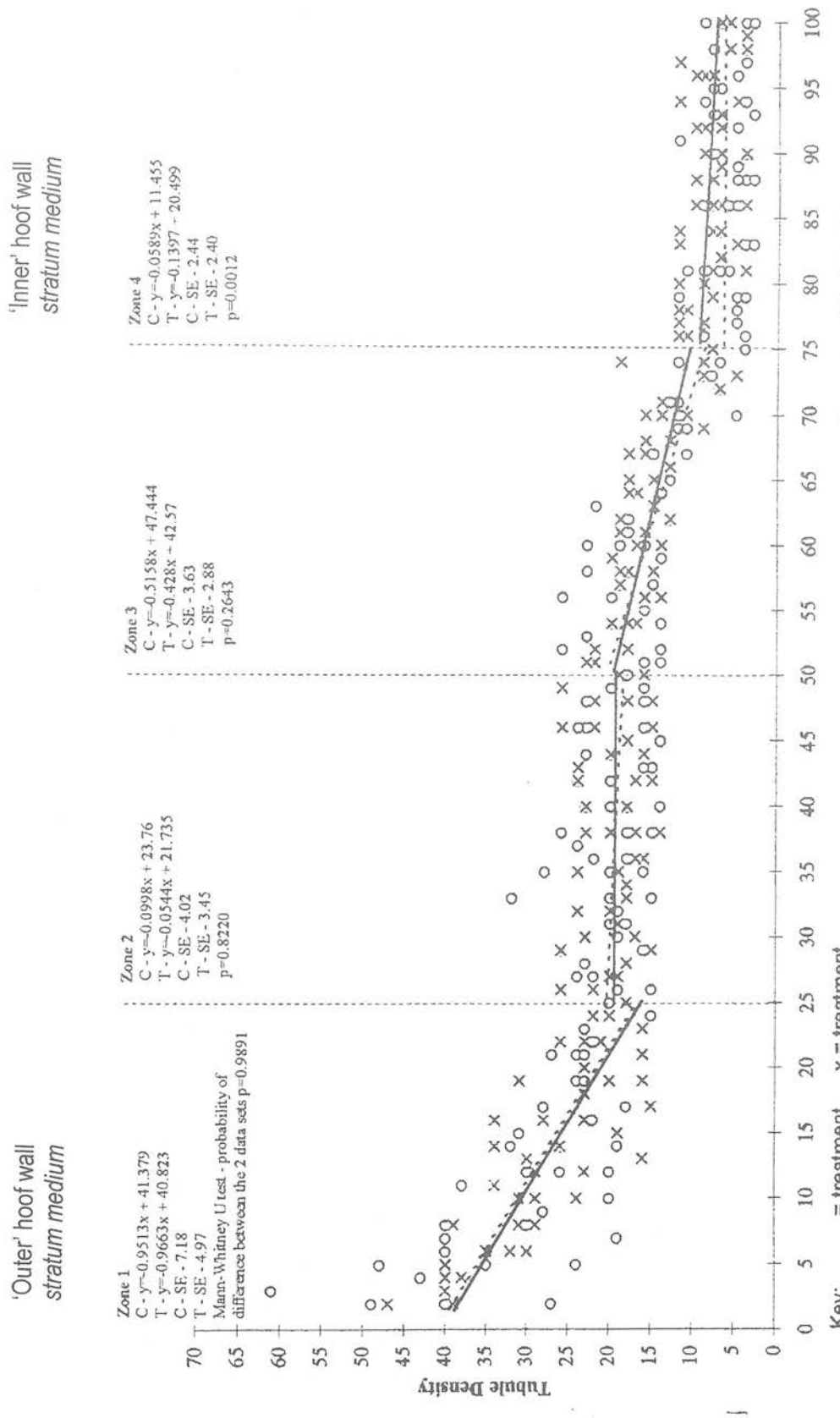
The data contributing to each zone of Figure 3.12 was analysed by comparison of regressions analysis.

There were no significant differences between Control and Treatment zonal data sets by comparison of regressions analysis.

Mann-Whitney U tests were also used to assess the differences between treatment and control group data for each zone.

There were no significant differences between treatment and control TDs in zones 1, 2 and 3 ($p>0.05$) by Mann-Whitney U tests, however, there was a significant difference in zone 4 ($p<0.01$) (see Figure 3.12).

Figure 3.12: Zonal arrangement of tubule density by percentage hoof wall depth



3.5 Discussion

3.5.1 Normal anatomical findings

Division of the SM of the hoof wall of these ponies into four separate zones on the basis of tubule density is proposed. For the pony samples investigated in this work, the objective division of the SM of the wall according to tubule density which is proposed in this thesis correlates well with previous workers' descriptive accounts which did not have supporting data:

Wilkens (1964) described 3 layers: an outer, a middle and an inner layer. These are probably equivalent to the zones 1, 2 and 4 described in this thesis. Stump (1967) agreed with these descriptions but also reported an 'intermediate layer' between Nickel's (1938, 1939) and Wilkens' (1964) inner and middle layers. This intermediate layer probably corresponds to the zone 3 which is proposed in this thesis.

The fact that the results from the selected and controlled groups of ponies in this work corroborates the descriptions of the wall tubules from previous workers, (who used samples from a wide variety of horses), lends weight to the possibility that the zonation described is a generalised equid pattern. Although 2 types of ponies were used in this work, the existence of a similar pattern needed to be shown in samples from other types of horses. In a follow-up study by Reilly *et al* (1998b), TD measurements were done, using the same methods described in Section 3.3.2 of this thesis, on twelvw LF hooves from a randomly-selected abattoir-killed sample of horses. In this case the method of sampling contrasted with that from the selected and controlled groups of ponies which had been used in the feeding-trial for this thesis, and yet the same, four-zoned pattern of TD was found in this sample too. This confirmed the existence of a quadri-laminar pattern in another equid type (See Appendix V).

However, the donkey hoof wall SM subjectively looks very different (Reilly 1997) and it remains to be seen what objective TD counting in the donkey hoof wall will reveal about its tubular distribution and the implications this has for the way the donkey hoof wall behaves mechanically (Reilly 1997). Given the more upright hoof conformation of the donkey compared to the horse and pony, studies of its hoof wall anatomy using TD counting, may reveal possible alternative methods of functional adaption, and this also illustrates the potential utility of comparative studies.

If data from hoof wll clippings from the bearing border also show the same pattern as for the more proximal hoof wall, then assumptions about tubule anisotropy

that others have made (Pollitt 1995, Greenough *et al* 1971) will also be supported. However, recent work (Hopegood, L personal communication 1999), with donkey hoof clippings, has shown discrepancies in tubule density at the distal site, probably as a wear artefact and further illustrates the difficulties of working with distal hoof clippings.

Another population of tubules is described as existing between, what is proposed as, zone 4 in this thesis and the SI. The existence of such tubules has been described by other workers such as Leach (1980), Bolliger (1991), Bragulla *et al* (1994) and Budras and Huskamp (1994). The work for this thesis does not report such tubules as a separate entity either because their density has made little impact on the data derived from the 'last cell' in the grid or because they have not been counted at all as a result of the discrepancy in alignment between the straight edges of the grid and the curvature of the SI.

Similarly, the degree of curvature of the SE surface will affect the outer zone tubule density readings as it will dictate how much material is missed before a count in the 'First cell' is possible. The method may well therefore underestimate the true mean tubule density of the SM by not detecting the full contribution that may be made by 'Zone 1'. Indeed, it may have omitted the possibility that the outermost tubules that occur in large numbers per sq mm of horn tissue may represent another population of tubules in the outer SM.

Such possible omissions are unavoidable unless a more sophisticated method of using curved counting cells is employed. Otherwise, counting ITDs in the curved outer portions of hoof tissue remaining, using straight cells, would result in magnified errors in extrapolating TDs from smaller ITD counts.

These factors should be borne in mind when interpreting tubule density data that is quoted between workers. The mean figure for tubule density derived from the untransformed data in this work is 17.5 mm^{-2} and is therefore in broad agreement with Bucher (1987). Transformation of the data shows that this figure is, however, an overestimate for the tubules counted. Care must therefore be taken not to quote mean values from non-normally distributed data. Such facts may become important in attempting to relate structural features to functional properties. In addition, from the

distribution of the density of tubules from Figure 3.11 and the zonation proposed above, care must also be taken to take account of possible sampling effects in the generation of tubule density data sets.

Bertram and Gosline (1986) explained that little headway had been made in understanding the functional significance of the tubular morphology within the hoof wall. Attempts at trying to relate the mechanical behaviour of the equine hoof wall to its striking tubular arrangement have demonstrated little direct relationship: Leach (1980) counted tubules, but not the distribution of tubules and could not demonstrate a relationship between stiffness and total tubule number in his samples. Bertram and Gosline (1986, 1987) and Thomason *et al* (1992) concluded that there was no consistent relationship between the major component of strain and the direction of the tubules, the intertubular material or the ground. They therefore concluded that the hoof is a multidirectional composite system.

However, with more consideration of zonation of tubules within the hoof wall and by exploring relationships between mechanical properties and structure within zones, then additional information about hoof function may emerge.

Leach (1980) postulated that during weight-bearing much of the ground reaction force is transmitted proximally up the wall (Thomason *et al* 1992). The high tubule density in zone 1 may allow stress to be concentrated in this part of the hoof wall as a function of its load bearing properties. The rapid decline in tubule density in zone 1 and the step-like pattern of tubule density found in zones 1-4 may be a mechanism for the smooth transfer of stresses from the SM to the SI and thus to the axial skeleton to which the SI is attached. Tubule density gradation across the wall could therefore be a mechanism for smooth energy transfer which would act in concert with the stiffness changes that are thought to be mediated by changing hydration levels across the wall as suggested by previous workers (Leach 1980, Leach and Zoerb 1983, Bertram and Gosline 1987).

Since energy is absorbed by separating 2 phases of a composite as a growing crack approaches (Cook and Gordon 1964) the tubule/intertubular interface in the composite structure of the hoof may be acting as a 'crack stopper'. The higher tubule densities in the outer zones may also be a protective mechanism for ensuring that

cracks do not propagate to the inner zones which are nearer sensitive structures within the foot. Such mechanisms will be modulated by water content within the zones of the hoof wall and by other features of the hoof's morphometry and chemistry such as those governing the strength of intercellular bonding. However, zonation of the SM, according to tubule density, may give the hoof wall the ability to act as a laminated structure. It is therefore proposed that a four zoned hoof wall may function as a quadri-laminar ply. The ability to shed an outer layer or zone of hoof wall that has contained a crack, as is often seen in horses (J.D. Reilly, personal observations), may in fact be a 'fail safe' mechanism that, paradoxically, protects the hoof capsule from catastrophic failure. Provided the lost material is close to the BB it can be replaced relatively quickly due to the mechanism of hoof wall growth. Other forms of hoof wall cracking that are seen may also in part be governed by the strength of the interface between zones within the hoof wall. Thus, this arrangement of tubules within the hoof may confer upon it the design advantages of a laminated composite.

Since the descriptive features of the tubules vary according to their location within the SM (Nickel 1938, 1939, Wilkens 1964, Bolliger 1991, Bragulla *et al* 1994), then tubule density may be used as a defining parameter for the location of a site of interest within the MDC SM. Objective investigation of the relationships between tubule density and other parameters such as tubule size, marrow and cortex size, and area fractions of tubular, tubular component and intertubular horn can then be carried out. These data are given in Chapter 4. Such findings can then be related to the results from mechanical tests on hoof. These data are given in Chapter 5. Using such data the morphological descriptions of previous workers can be extended into the field of biomimetics as Vincent (1992) has envisaged.

In addition, we have a means of comparing the tubular distribution within the SM of the hoof wall between species. If similar patterns of zonation exist, or do not exist, in the hooves of other species or in any other biological structures then this will be a source of further information about function.

3.5.2 Biotin effects on tubule density

The two methods of analysing the zonal TD data which contributed to Figure 3.12 gave interesting differences:

Mann-Whitney U testing revealed a significant difference between Control and Treatment data for TD in zone 4 only. However, comparison of regression analysis had shown no differences in this, or any other zone. This shows that although the distribution of the tubule density data may not be different in zone 4, the absolute values are. Thus a comparison of regression may not show a difference in the distribution of the data but by substituting a value of say, 85% HWD for y in the regression equations for Control and Treatment in zone 4, respective TDs of approximately 4 tubules/mm^2 and 5.5 tubules/mm^2 are derived.

Furthermore, visual inspection of Figure 3.12 reveals that the probability of the difference between the two data sets being insignificant diminishes as one moves progressively from zone 1 to zone 4. Although the significant differences is given for zone 4, the difference is most marked in the transitional area between zone 3 and zone 4. Similar substitution of figures into the regression equations testifies to the increased difference between the two data sets at this point as shown by the regression lines being most divergent here.

There are two reports in the literature of biotin supplementation having brought about an increase in tubule density: The first was Kempson *et al* (1989) with pigs, and the second was Dittrich *et al* (1994) with horses. In neither case are the methods sufficiently described to be able to make a comparison as to whether the changes occurred in similar parts of the SM of the hoof wall as the effects seen in this work. However, the effects described in these cases must have been far less subtle than those found in this work as these authors describe differences in overall mean TD between treatment and control animals which were not apparent in this work.

How the difference in TD found in this work could have been bought about in zone 4 only cannot readily be explained. For an absolute increase in TD to have occurred, presumably, more papillae would have to have been produced at the corium. It is not possible to assess whether this is the case and it is recommended that future work allows for being able to count papillae either by dermo-epidermal separation before storage, or by using stereological techniques to count their presence from histological slides. Until this can be assessed, it can only be speculatively assumed that biotin may be a limiting nutrient for papillae or tubule formation. It is

interesting to note that Dittrich *et al* (1994) only report a change in TD at their highest dose rate of 40mg per day. Further work to assess the effects of differing doses of biotin is suggested to assess whether this finding can be corroborated.

An alternative explanation for the treatment effect may be that biotin supplementation to the papilla decreases the chance of 'verdamman' or tubule fadeout, or that 'budding' of the papillae to produce more than 1 tubule may have occurred . Again, stereological counting would be required to assess this.

A further alternative may be that the increase in TD may be relative, and that the intertubular horn area has diminished in this zone. This is addressed in Chapter 4.

3.6 Conclusions

A quantitative method for measuring tubule density was used to assess this parameter on MDC material from the LF foot of ponies in the controlled feeding trial.

The stratum medium was found to consist of four distinct zones as defined by tubule density. This is new, quantitative, anatomical information and it is proposed that the hoof wall may act as a quadrilaminar ply.

This existence of four zones of tubules has also been confirmed from tubule density measurements performed on feet from a randomly-selected abattoir-killed sample of horses. (See Appendix V).

There was an effect of biotin supplementation on tubular density with treatment animals having a significantly higher tubule density in zone 4 of the SM ($P<0.01$ by Mann-Whitney U Test).

ADDENDUM TO CHAPTER 3

Introduction

The purpose of this addendum is to give further information about the follow up study into tubular density (TD) at the midline dead centre (MDC) of the hoof wall of horses which was conducted by Reilly *et al.* (1998b), and referred to in Section 3.5.1, page 142, of this thesis.

The work for the PhD thesis had been conducted on material from a selected and controlled group of **ponies** which had been used in a feeding trial. Investigation of tubule density (TD) at the midline dead centre (MDC) of the hoof wall of these ponies had revealed a distinct and stepped four-zoned pattern of TD by hoof wall depth (HWD). (See Figure 1 below).

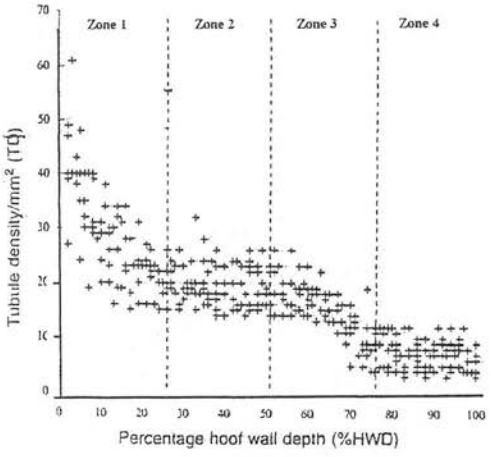


Fig 1: Tubule density (TD) by percentage hoof wall depth (%HWD) to show the 4 zones of the stratum medium *at the midline dead centre for ponies (after Reilly* et al. 1996).

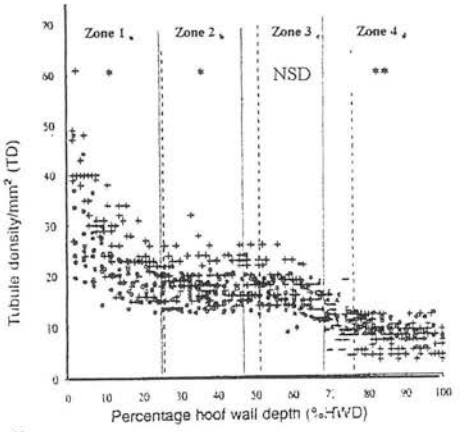


Fig.2: *Comparison of tubule density (TD) and zonal boundaries by percentage hoof wall depth (%HWD) for the* stratum medium *of ponies and horses.*

In order to test whether or not the same mean value and pattern of TD in the MDC hoof wall material existed in the wider equine population, it was decided to conduct a study of TD at the MDC for other equids from a randomly selected, abattoir-killed, population of **horses**. This work was undertaken with colleagues at De Montfort University, Leicester. The proposal for the research was made by J D Reilly and the practical elements of the work were carried out by two students, S N Collins and L Hopegood. The research was supervised by J D Reilly together with Professor R J Latham and Dr B Cope of De Montfort University.

Materials and Methods

The left fore feet of 8 randomly selected slaughterhouse horses were used to provide hoof samples. The sampling site was the midline dead centre of the *Stratum medium* of the hoof wall after Reilly *et al.* (1996).

The centre point of the sample was taken as the mid point between the coronary band and the bearing border. The final block taken from the hoof capsule was 1 cm in height, 1 cm long (mediolaterally) and encompassed the full dorso-palmar extent of the stratum medium of the hoof wall. Reilly *et al.* (1996) referred to this as the full hoof wall depth of the *Stratum medium* (HWD). The sample was taken at right angles to the direction of the tubules. A sledge microtome was used to cut a horizontal section of 12 μm from the centre of the block. Sections were stained with haematoxylin and eosin, dehydrated and mounted in DPX with a coverslip. Digitised images of each section were captured using a video camera and Global Laboratory software. Images were processed using NIH Image (Public domain software version 1.59) to enhance tubule definition. The digitised images were enlarged using Adobe Photoshop to A3 format, prior to printing. A grid was then overlaid on the image and a tubule density count was carried out according to the method of Reilly *et al.* (1996).

Statistical Analysis

The results were analysed using Minitab. Graphs were produced using Minitab and Excel. The normality of data was established using the Kolmogorov-Smirnov normality test. Differences between zones for transformed data were analysed using one way analysis of variance (ANOVA) and Tukey test. Zonal tubule density comparisons between horse and pony were evaluated by *t* test using transformed data.

Results

1. A similar four zoned pattern of tubule density by HWD was found for the abattoir-killed horses, as it had been for the ponies. However, the zonal boundaries of the TD curves varied between ponies and horses. (See Figure 2 above).

2. The values for TD within each zone were also found to differ. (See Table 1 below).

Table 1: Comparison of tubule density and zonation in the *stratum medium* at the midline dead centre (MDC) for horse and pony hoof.

	Horse		Pony (Reilly *et al.* 1996)	
Zone	% HWD	Tubule density (tubules/mm^2)	% HWD	Tubule density (tubules/mm^2)
Zone 1	0-25	>22	0-26	>27
zone 2	25-47	16-22	26-51	16-27
Zone 3	47-69	11-16	51-77	8-16
Zone 4	69-100	<11	77-100	<8

Conclusions

The major conclusions from this piece of work were:

1. Ponies and horses have a similar four zoned pattern of TD distribution within the MDC SM.

2. The precise boundaries between zones differ between the ponies and horses.

3. The overall mean TD for the whole HWD is similar between ponies and horses at 16 tubules/mm^2.

4. Mean TD *within* zones varies between ponies and horses for zones 1, 2 and 4 but not for zone 3.

5. TD values for zones 1 and 2 for the pony were significantly higher than those for the horse ($p<0.05$).

6. TD values for zone 4 for the pony were significantly lower than for the horses ($p<0.01$).

The question as to whether horses and ponies have similar TD arrangements within the SM at the MDC had been answered. They do; but there are interesting differences in detail, and interesting similarities in zone 3, which require further investigation in order to understand their functional significance.

CHAPTER 4

Morphometry of the *Stratum medium*

4.1 Introduction

The review of the literature revealed that there is debate about the role of the tubular and intertubular arrangement of hoof horn, about the relative contribution of tubular and intertubular horn to the whole, and the effects of biotin on these components of the *stratum medium*.

In those studies that have carried out morphometric measurements on tubular and intertubular features of the SM, results vary: Bucher (1987) calculated that tubular horn accounted for 60% of the area fraction of the inner zone of the SM and for 75% in the outer. Area fraction data for tubular horn from the inner zone of the SM reported by Pellmann *et al* (1993) of 33.3% are in marked contrast with the 60% recorded by Bucher (1987), and so this feature of the composition of the hoof wall needs further investigation.

Geyer (1980) stated the importance of evaluating tubular morphometry at a given position within the hoof wall in order to take into account the apparent differences in size of tubules at different radial sites around the hoof capsule. These differences in morphology of the SM around the hoof capsule have been observed (J.D. Reilly - unpublished observations) and the opinion given by Geyer (1980) has been heeded in the selection of the specific site for sampling for the work in this thesis.

4.2 Aims

The aims of this chapter were to:

(i) obtain hoof wall material of known history that had been generated under strictly controlled experimental conditions within a trial group selected from an homogenous equine population;

(ii) obtain this material from accurately defined anatomical sites both from the hoof capsule (at the MDC site) and within the *stratum medium* (sampled from mid zones);

(iii) develop methods for objective measurement of tubular and intertubular components of wall horn and use these to;

(iv) obtain normal values for tubular and intertubular components of wall horn from the experimental ponies;

(v) assess whether biotin supplementation had any effect on these values.

Specifically this required the measurement of:-

- The absolute area measurement of the marrows, cortices and tubules.
- The area fractions of marrows, cortices and tubules.
- The area fractions of tubular and intertubular horn and tubular: intertubular area fraction ratio.

(vi) to evaluate this information in terms of the current understanding of the hoof wall and how it functions.

4.3 Materials and Methods

4.3.1 Development of Methods

Histomorphometry, that is the objective measurement of anatomical features from prepared slides of histological material, has provided the means for quantifying features of biological structures (Weibel 1979). However, its practical application to studies involving the assessment of histological sections, has been limited.

Traditionally, such studies have had to rely upon manual measurements taken from either projected images or photomicrographs, with measurements often being restricted to ratio quantification derived from point counting operations. Such work is very time consuming, painstaking and costly (Weibel 1979). However, the advent of computer technology has made semi-automated quantification a more efficient and practical option. Recent developments now provide a cost-effective alternative to manually-based measurements systems.

Computer-based imaging has the potential to significantly improve reliability and repeatability and also to reduce the procedural time, especially in those situations where photographs were needed (Gatlin *et al* 1993). These advances also make planimetry studies, in which area fraction and linear measurement are determined, possible. In addition, computer programming allows automation of many of the procedural operations (Weibel 1979), minimum observer 'sampling bias' (Leach

1996), and enhanced image production for the observer, prior to any measurements being taken.

In the light of these advantages, a computer-based image analysis system was developed to allow semi-automated quantification of hoof horn parameters.

4.3.2 Preparation of hoof wall sections for morphometry

Sections were cut at 10μm as for TD cutting in Section 3.3.2 in Chapter 2, but this time stained with Alcian Blue (Ab), Periodic Acid (PA), Schiff's Reagent (S) (AbPAS) using the following protocol:-

AbPAS Staining Protocol (from Geyer, H. personal communication 1992)

Ab – 5 min.

Rinse with distilled water.

PA – 2 min.

Rinse with distilled water.

S – 10 min.

The AbPAS preferentially stains the mucopolysaccharides or complex carbohydrates associated with the intercellular material (ICM) and the cell margins to give very good cellular margin definition. This makes these stains ideal for preparing sections for morphometric area measurement analysis (Hofstetter 1985) as it delineates the cellular boundaries (see Figure 4.1). This is different to the H&E stain, which is used for preferentially staining the marrow cavity for point counting, as used for TD assessment as described in the previous chapter.

4.3.3 Determination of within zone sampling sites

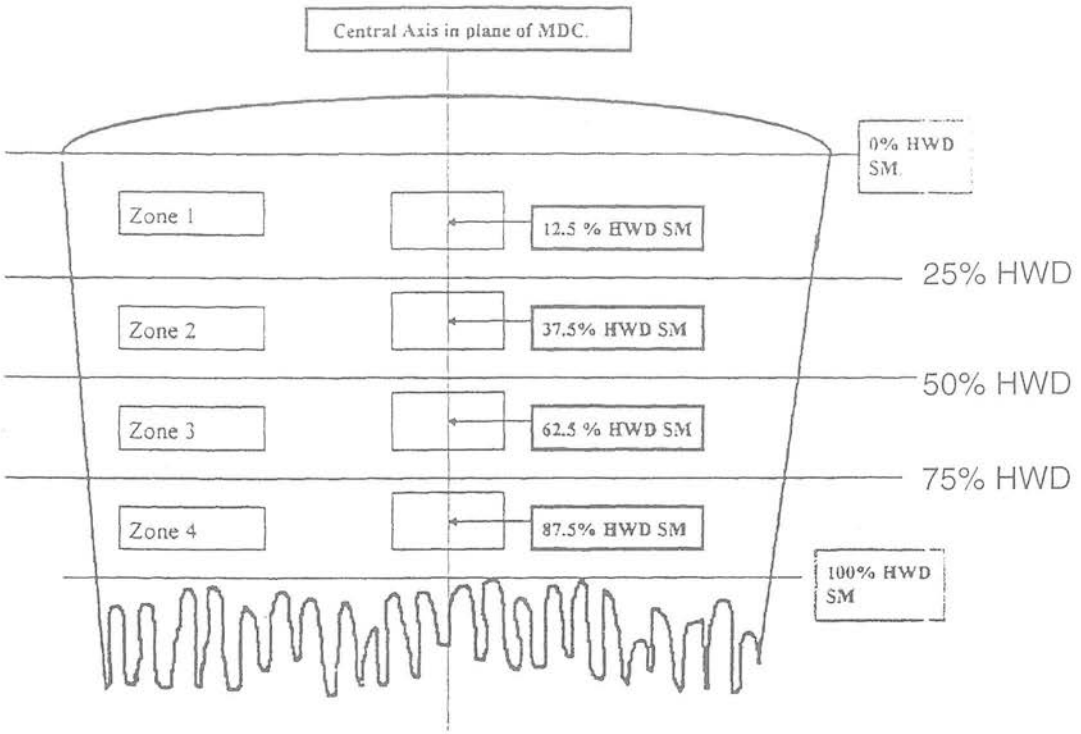
An inherent problem exists with regard to area fraction measurement. In order to be able to define accurately the tubule boundary, high power magnification is required. However, by using high power objectives, the field of view within each zone is reduced.

It was decided to sample at the mid-point of each of the hoof wall zones established in Chapter 3. Thus, sampling for morphometric measurement took place at 12.5% , 37.5%, 62.5% and 87.5% of HWD (see Figure 4.2). Thus, for each full

Figure 4.1: High power magnification of hoof horn tubule stained with AbPAS to show cell boundaries

100µm

Figure 4.2: Mid zonal sampling points within the stratum medium for morphometric measurements



Key
SM: stratum medium
HWD: hoof wall depth
MDC: midline dead centre

HWD section for morphometry from the LF of each pony, 4 zones were measured giving 4 x 8 = 32 sampling sites from all 8 experimental ponies.

4.3.4 Statistical Analysis

Statistical Analysis of the data set was conducted using Minitab version 11.0 for Windows (Minitab corp. USA) and Excel Version 5.0 (Microsoft Corp USA).

4.3.4.1 Normality Assessment and Testing

The data set was assessed for normality by using Nscore (Minitab Corp USA) correlation plots.

Normality testing was used in order to determine the appropriate subsequent statistical tests that could be used. Where normality was indicated, parametric testing techniques were employed. Conversely, if the data distribution was non-normal, then the equivalent non-parametric test was used. The data for the individual zones and horses were stacked in order to produce group data for statistical analysis.

4.3.4.2 Statistical Manipulation of Data

Absolute area measurement comparisons were conducted by group. Marrow analysis was conducted using a parametric one-way analysis of variance test (ANOVA) with "between group" comparisons by Tukey testing at a confidence interval of 95%. The Kruskal-Wallis Test (non-parametric equivalent of ANOVA) was used for cortical and tubular comparisons.

4.3.5 Hoof wall morphometry

Images of the AbPAS stained sections were captured by a microscope-mounted Sony digital camera to produce 256 grey scale levels. The image was fed to the public domain software "Image", version 1.59 (National Institute of Health, USA), which was loaded into a MacIntosh 8500 computer with 8 MB RAM. Preliminary use of the system revealed that, in common with many biological materials, there was insufficient grey scale contrast between the structural features to permit automated object discrimination. Thus a manual approach to delimiting the borders of the tubules was employed.

In order to be able to define accurately the structural features of the hoof wall, high power magnification was required. However, higher power magnification reduces the number of features within a given field. Hence, a compromise had to be

reached between magnification levels required to permit feature discrimination and feature numbers required for the sample to be considered representative of the structure.

Initial image acquisition was performed with a 4 x objective and a 3.3 x photo-eyepiece. This, combined with a 1.25 x gain arising from the configuration of the microscope head, resulted in a total magnification of ~x20.

4.3.5.1 Pixel calibration of the image analysis system

The image analysis system was calibrated with respect to the total magnification using the captured image of a scaled graticule. A pixel count of a known graticular distance was made both in the horizontal (x) and vertical (y) directions. This enabled two important calibration calculations to be achieved. Firstly the pixel count per unit length and secondly, the aspect ratio. The aspect ratio is the ratio between the pixel count per length in the x direction compared with that in the y direction.

Knowing the pixel calibration and the measured distance to the zonal mid points calculated from the photographic images, pixel co-ordinates for each sample site were determined. The microscope stage was moved in the y direction until the pixel counter corresponded with the calibrated mid-point value.

The x, y co-ordinates were zeroed to this position. From the known HWD from the photograph for any given sample, and the pixel calibration value, the value of the mid point sampling sites was determined in terms of pixel depth, and the microscope stage accurately positioned to these sites.

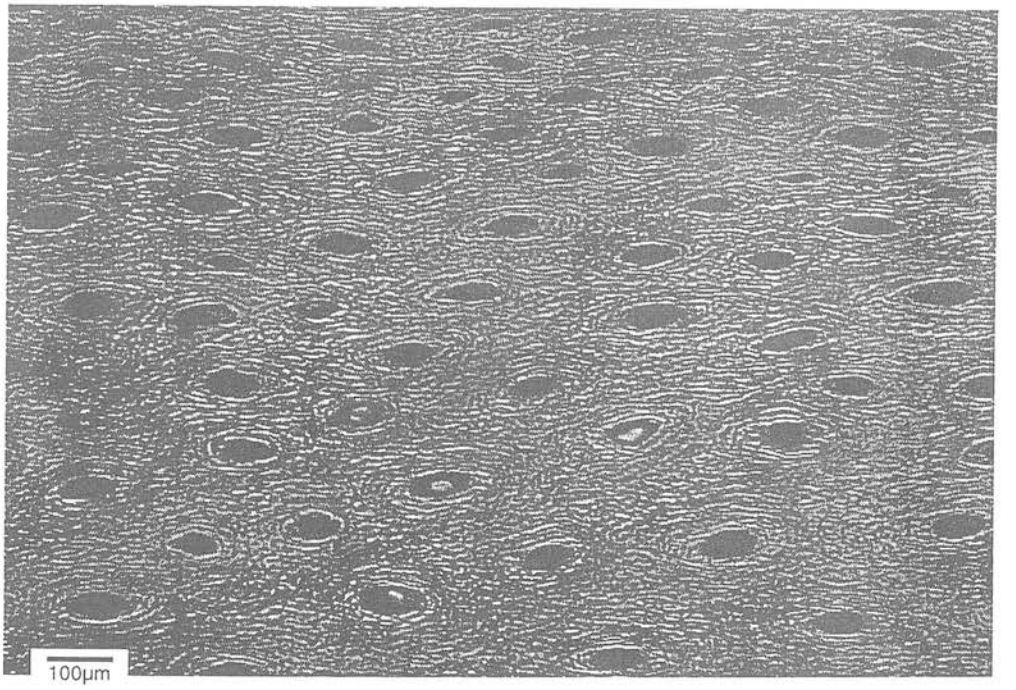
4.3.5.2 Correction procedure for variation in light intensity

As uniform illumination is recognised to be an important determinant in accurate boundary definition, a 'blank field' capture was performed. The resultant represents uniform white light. All subsequent images were then displayed in 256 grey scale relative to this baseline. In this way it was possible to control for variations in light intensity within the microscope.

4.3.5.3 Morphometric data collection

The initial captured image (see Figure 4.3) was imported into the NIH-Image program and subsequently analysed through the computer interface. Images were

Figure 4.3: Initial capture of stratum medium image



enhanced prior to analysis in order to improve edge detection, which helped with manual discrimination. A standardised procedure of 1x 'smooth' and 2x 'sharpen' automatic procedures from the software menu was adopted to ensure consistency in pre-processing operations. Once this had been completed, a three stage semi-automated method was used that provided both feature-specific (absolute area measurements) and field-specific (area fractions) data (Weibel 1979) for the hoof wall. This included absolute area measurements of marrow, cortex and tubule, and area fraction information for the tubular and intertubular components.

4.3.5.3.1 Stage 1: Marrow Imaging

The internal margin of the tubule cortex was used to delimit the tubule marrow cavity. Discrimination of the marrow was achieved by outlining this margin using the free-hand drawing tool. The enclosed area was then "shaded" by allocating an arbitrary grey value from the Look Up Table (LUT) grey scale (See Figure 4.4).

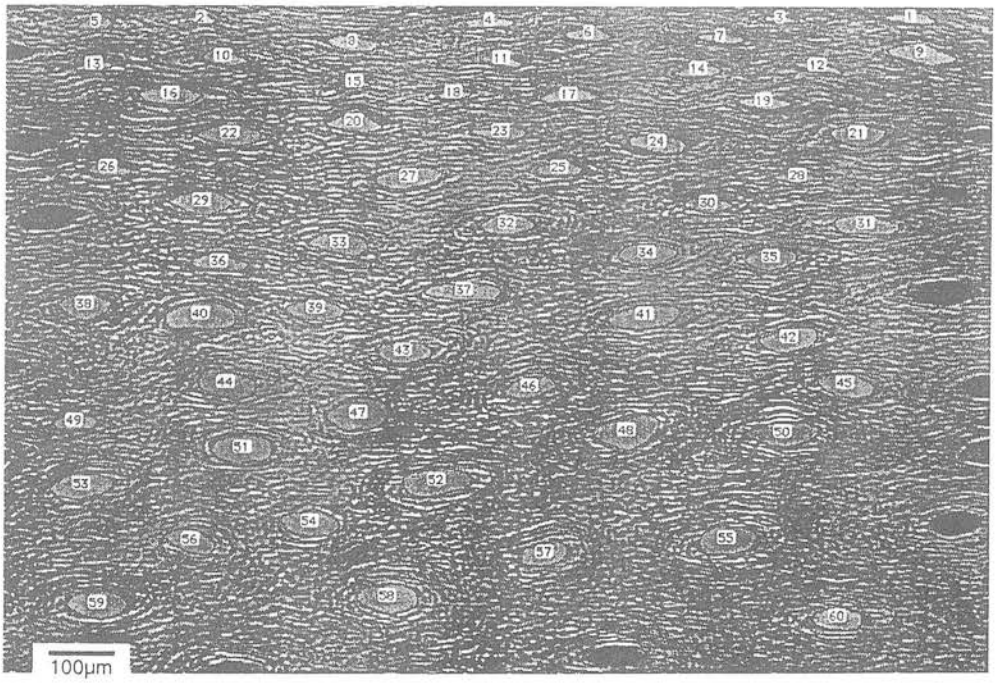
The resultant image was segmented, using the "Density Slice" processing function, to leave only areas possessing the grey scale value selected in the shading process for further analysis and measurement. Hence the resultant image contained not only the desired 'true particles' i.e. the marrows features, but also other false particles of the same grey value. Two methods of image editing were employed in order to remove these 'false particles' prior to measurement.

Firstly, particle discrimination limits were set such that only objects measuring between 99 - 9999 pixel units were selected. In addition, the images were eroded and dilated, three times. The resultant images were numbered using the labelling option in preparation for measurement. A copy of this image was stored on hard disc, prior to measurement.

4.3.5.3.2 Stage 2: Tubule Imaging

The outlining procedure was repeated for the external tubule cortex margin. The interface between the outer edge of the tubule cortex and the start of intertubular horn was not always readily discernible. Hence, the external margin had to be determined on a "discretionary basis". Those cells whose margin arched around the tubule were considered to be cortical, whereas those whose margins arched away

Figure 4.4: Measurement of marrow absolute areas



from the tubule, were judged to be intertubular horn. On this basis, the tubules were delineated. Once this had been completed for each tubule, the object was shaded as for the marrow. The same segmentation and discrimination procedures as per stage 1 were adopted to separate 'true' from 'false' particles, prior to labelling (See Figure 4.5).

4.3.5.3.3 Stage 3: Total Area of Image

The total area of the image was calculated by outlining the perimeter of the image. In order to take account of "edge effects" only those tubules that completely fell within the image boundary were considered as 'true particles'. Part tubules at the edge of the field of view were disregarded, and considered as 'False Particles'. The enclosed area was shaded and labelled as described previously. It is important to note that this edge effect will result is an over-estimation of the intertubular horn area fraction, as it is not possible to quantify the area of intertubular horn associated with the part tubules. (See Figure 4.6).

4.3.6 Measurement

The criteria selected were absolute area measurement of marrow, cortex and tubule, and area fraction of marrow, cortex and tubule, as well as tubular:intertubular horn ratio.

Having previously established pixel calibration criteria, all area measurements were automatically converted by the computer program to μm^2. Having established the marrow and corresponding tubule area measurements, the cortical area values were obtained by difference. The resultant data were stored for subsequent analysis. Figure 4.7 summarises the procedure for absolute area measurement of hoof horn parameters within the hoof wall.

4.3.7 Semi-Automation

Following the successful development of this technique a macro programme was written to automate the image editing and measurement procedures, following the manual discrimination of the structural components (see Appendix III). The use of this macro programme significantly reduced the procedural time.

Figure 4.5: Measurement of tubule absolute areas

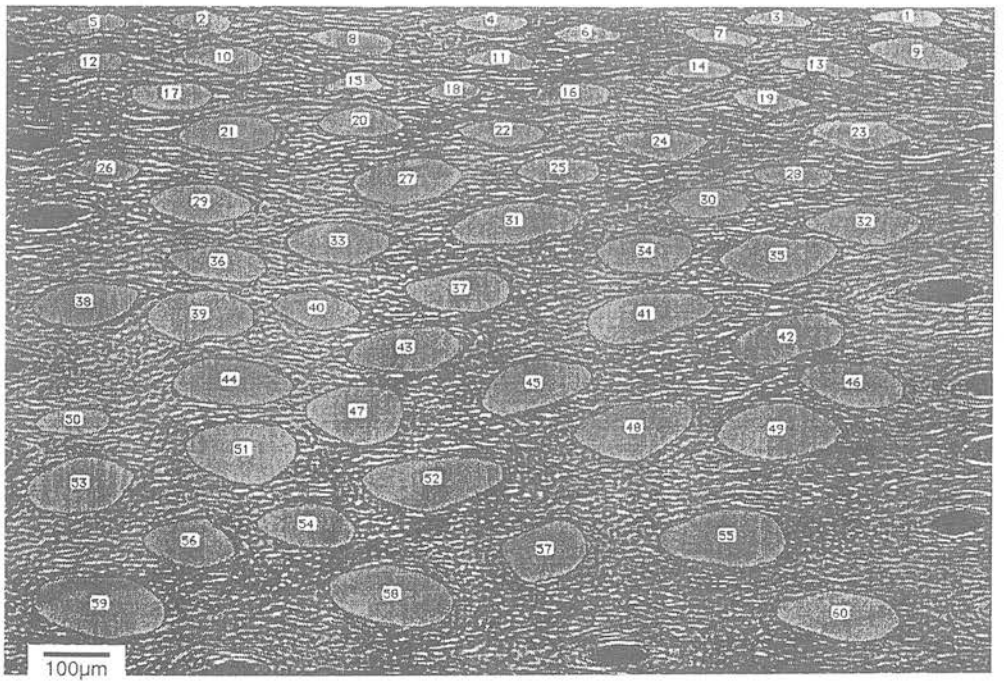


Figure 4.6: Measurement of total absolute area of field

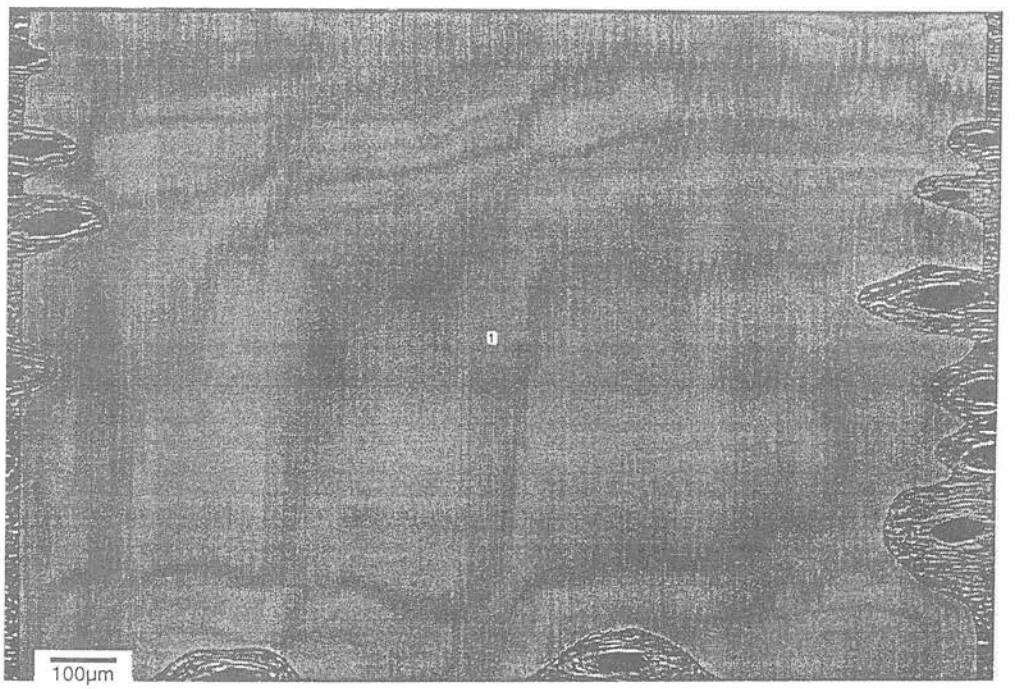
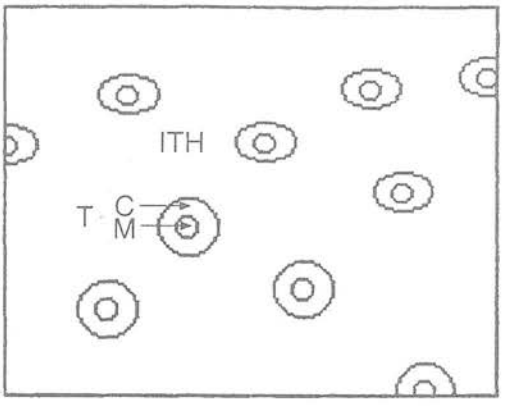
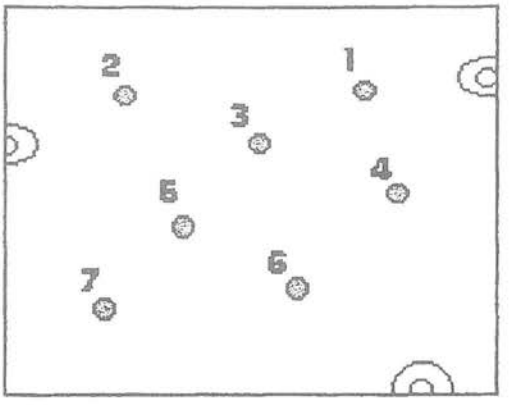


Figure 4.7: Summary of procedures for area measurement of hoof wall components

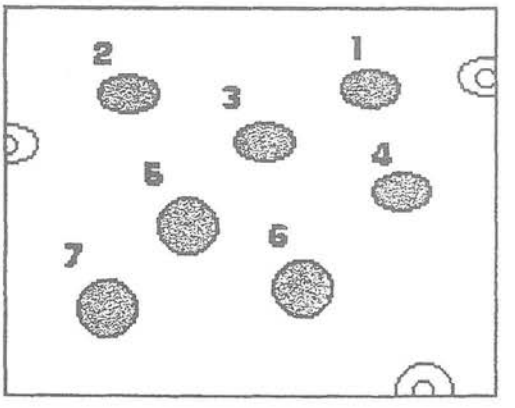
1) Initial capture of stratum medium image



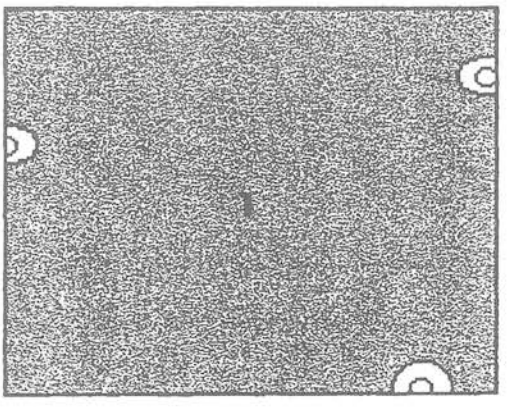
2) Measurement of marrow absolute areas



3) Measurement of tubule absolute areas



4) Measurement of total absolute area of field



Key

M - Marrow
C - Cortex
T - Tubular horn
ITH - Inter-tubular horn

4.4 Results

Total Area Measurements and Total Tubule Counts

The total area of hoof wall sampled from the 32 sampling sites from all 8 ponies was 49,749,673 μm^2. The Treatment group accounted for 49.7% of the total area and the Control group, 50.3%. The zonal area breakdown by individual pony, and by group is shown in Tables 4.1 and 4.2 respectively, below:

Table 4.1: Area measurements of sampling field by pony by zone in μm^2

Pony	Zone 1	Zone 2	Zone 3	Zone 4
Control 1	1611870.00	1607372.12	1569591.38	1448041.38
Control 2	1609395.38	1604366.12	1508701.25	1466143.75
Control 3	1622216.12	1568914.12	1618634.62	1614167.75
Control 4	1593692.50	1592661.00	1550935.62	1414143.25

Pony	Zone 1	Zone 2	Zone 3	Zone 4
Treatment 1	1451175.75	1595888.25	1611507.06	1553835.38
Treatment 2	1517396.22	1553795.62	1583319.75	1563451.06
Treatment 3	1554114.28	1623088.25	1496340.88	1608881.75
Treatment 4	1442764.38	1593143.50	1566541.12	1433587.00

Table 4.2: Area measurements of sampling field by group by zone in μm^2

Group	Zone 1	Zone 2	Zone 3	Zone 4
Control Group	6437174	6373313	6247863	5942496
Treatment Group	5965451	6365916	6257708	6159755

The total areas measured for Control and Treatment groups is given in Table 4.3 below:

Table 4.3: Total areas measured in sampling field by group

Group	Total area measured
Control Group	25000843 μm^2
Treatment Group	24748830 μm^2

A total of 1,000 measurements of each of marrow, tubule and cortex were made.

A total of 488 tubule measurements were recorded from the Control group and 512 from the Treatment group. The total number of tubules counted by zone is given in Table 4.4.

Table 4.4: Tubules counted per zone

Control Group	Zone			
	Zone 1	Zone 2	Zone 3	Zone 4
Control 1	56	26	21	18
Control 2	32	27	20	13
Control 3	58	41	34	15
Control 4	47	38	29	14
Treatment Group				
Treatment 1	33	26	28	11
Treatment 2	48	30	25	19
Treatment 3	61	48	24	24
Treatment 4	43	36	37	18

4.4.1 Cortical area Measurements

The cortical area measurement represented the difference between the tubule area measurement and the corresponding marrow area measurement. Cortical area measurements were determined by subtracting the marrow values from the corresponding tubule value.

4.4.2 Basic descriptive statistics

Normality testing was conducted for tubules, cortices and marrows area data by groups. Based upon the "n scores normality test" (Ryan *et al* 1976), the distribution of marrow area data in both the control and treatment groups was found to be normal. However, the 'n scores' normality plot for tubule and cortex data indicated that the data was non normal for both control and treatment groups.

Basic descriptive statistical information for treatment and control group absolute area measurements are given in Table 4.5 below:

Table 4.5: Mean absolute area measurements by Group

Group	Mean (μm^2)	Standard Deviation	Range	Coefficient of Variation
Control Group				
Marrow	2024	±858	456 - 5454	0.42
Tubule	12186	±8927	2546 - 77540	0.73
Cortex	10359	±8929	770 - 75300	0.82
Treatment Group				
Marrow	1852	±713	172 - 5963	0.38
Tubule	11887	±8504	1771 - 59048	0.72
Cortex	9917	±8929	220 - 55883	0.82

4.4.3 Comparison of absolute area measurements for treatment and control groups

Frequency histograms for Control group tubule cortex and marrow absolute area measurements are given in Figures 4.8, 4.9 and 4.10. Frequency histograms for treatment group marrow, tubule and cortex absolute area measurements are given in Figures 4.11, 4.12 and 4.13.

A significant statistical difference ($P<0.01$) was found between the marrow sizes in the Treatment and Control groups by ANOVA. The treatment group had a significantly smaller mean marrow size of 1852 μm^2 ±713.5 compared with mean control marrow size of 2024 μm^2 ± 858.2 (see Table 4.5 and Figures 4.10 and 4.11).

Figure 4.14 superimposes the control and treatment marrow area measurements frequency histograms to show the shift in treatment marrow size and Figure 4.15 is a bar chart to show the group differences in marrow cortex and tubule area measurements.

No significant differences at the 95% confidence interval, were detected between the absolute area measurements in either the cortical or tubular measurements for the Control and Treatment groups ($P>0.05$) (See Table 4.5).

Figure 4.8: Frequency histogram of control group tubule area measurements

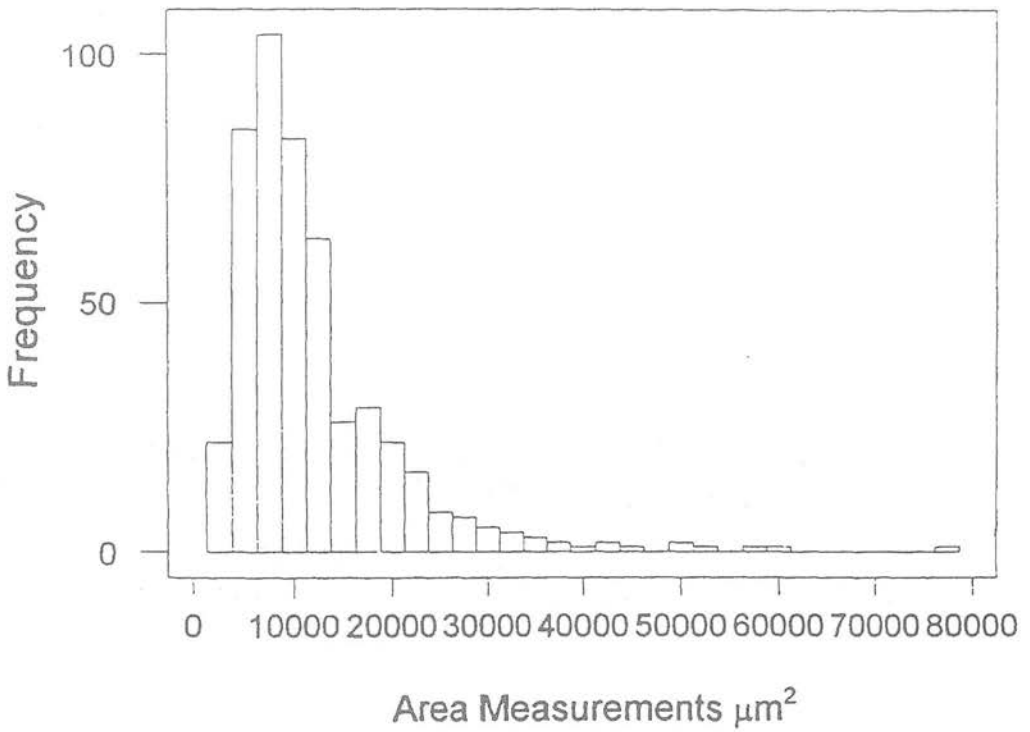


Figure 4.9: Frequency histogram of control group cortex area measurements

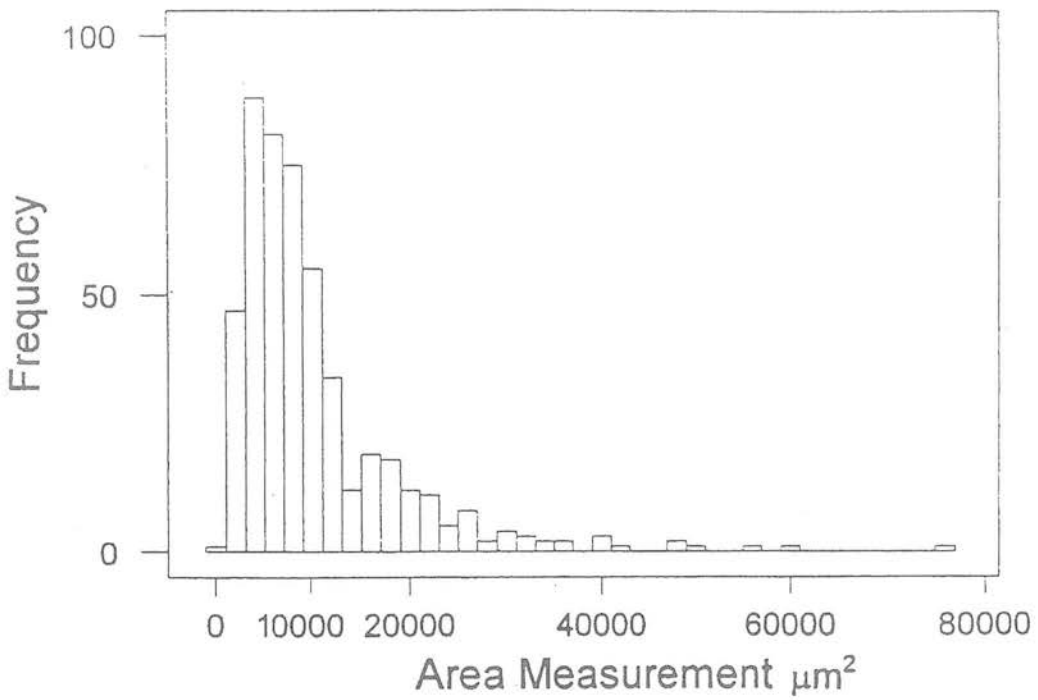


Figure 4.10: Frequency histogram of control group marrow area measurements

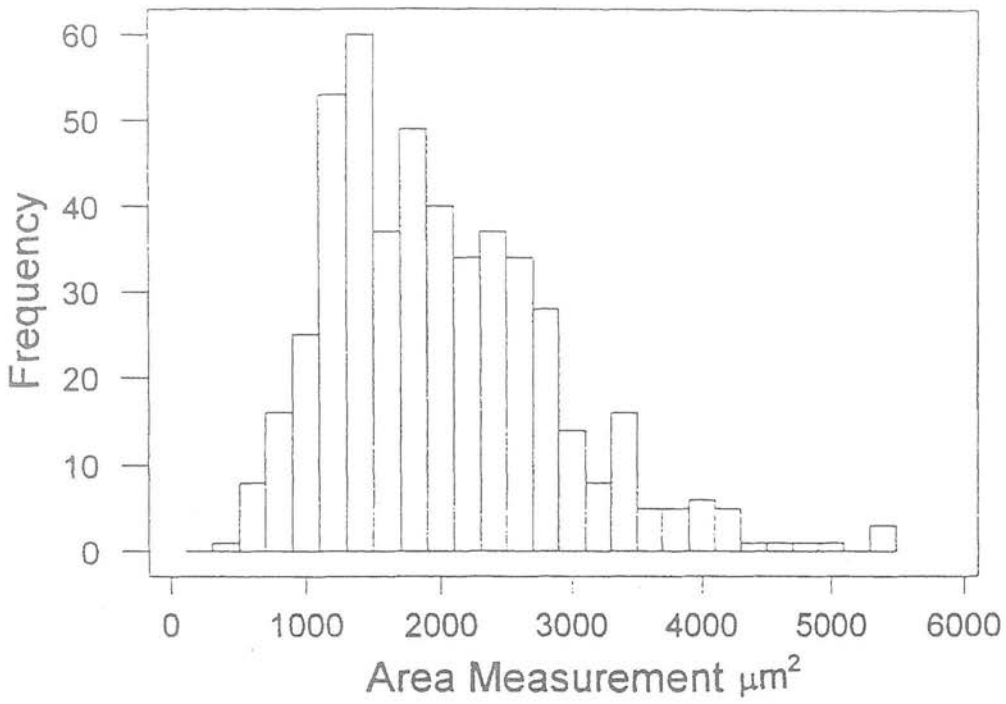


Figure 4.11: Frequency histogram of treatment group marrow area measurements

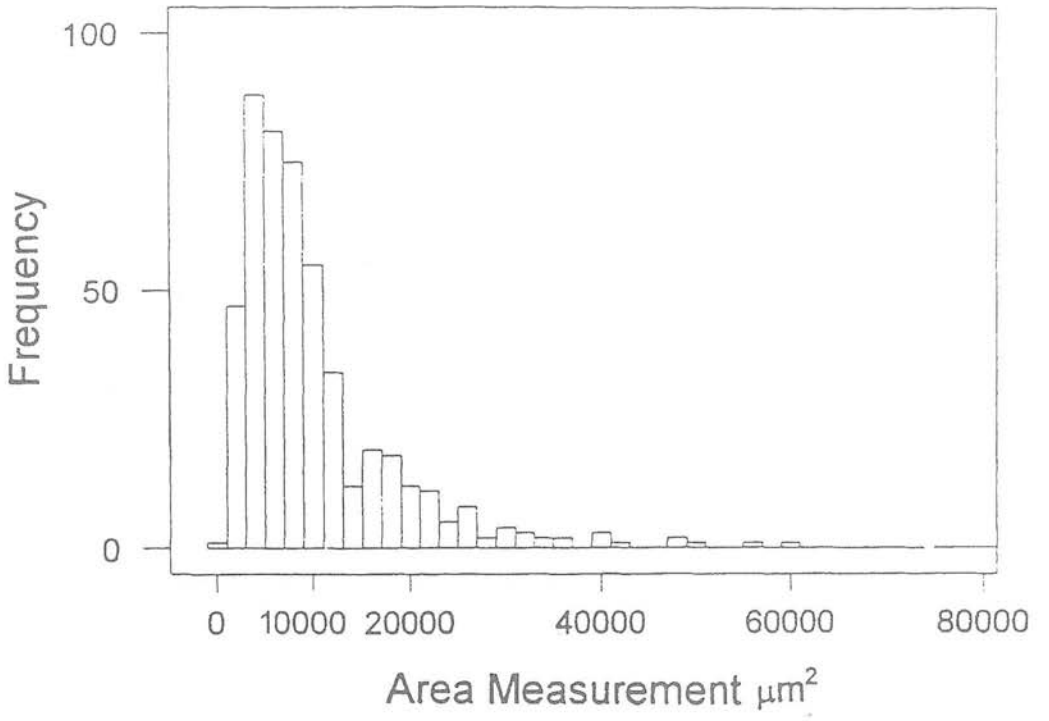


Figure 4.12: Frequency histogram of treatment group tubule area measurements

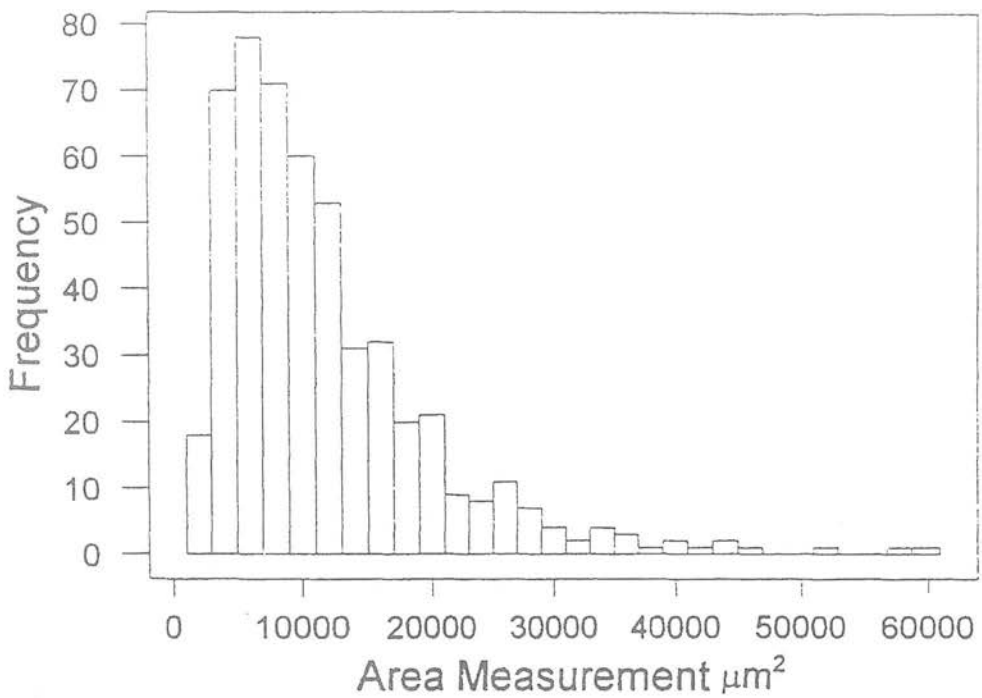


Figure 4.13: Frequency histogram of treatment group cortex area measurements

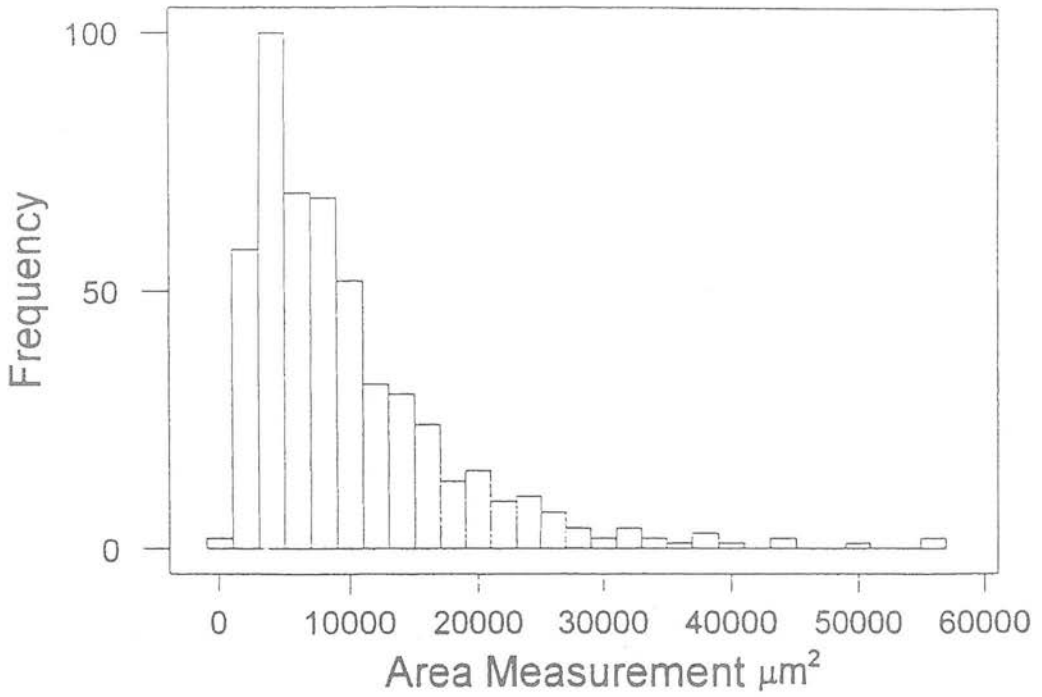


Figure 4.14: Frequency histogram of marrow area measurements for control and treatment groups

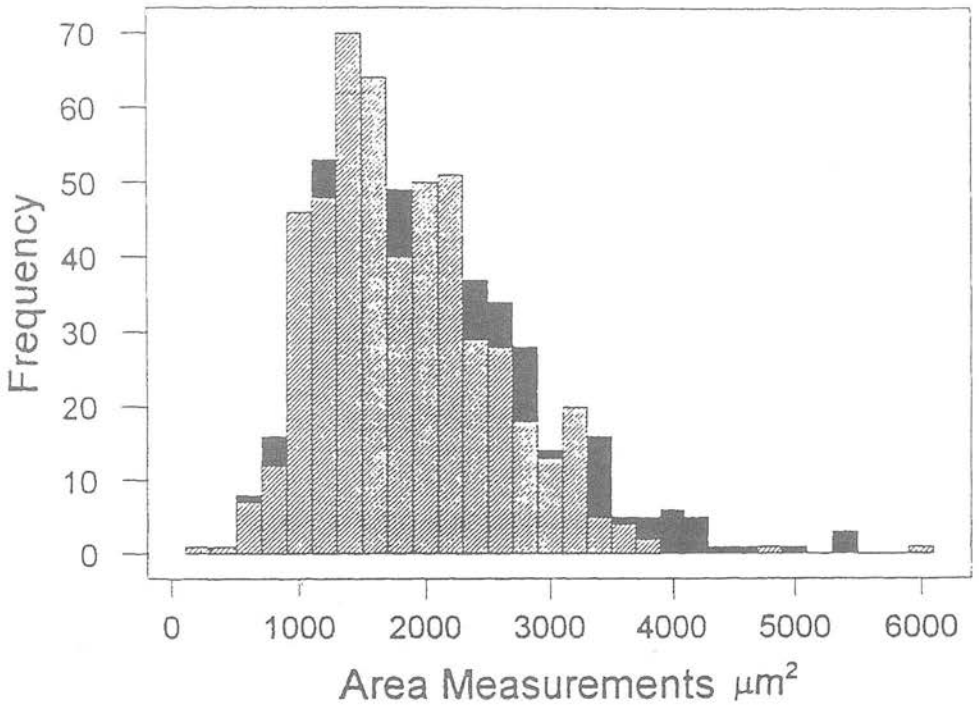
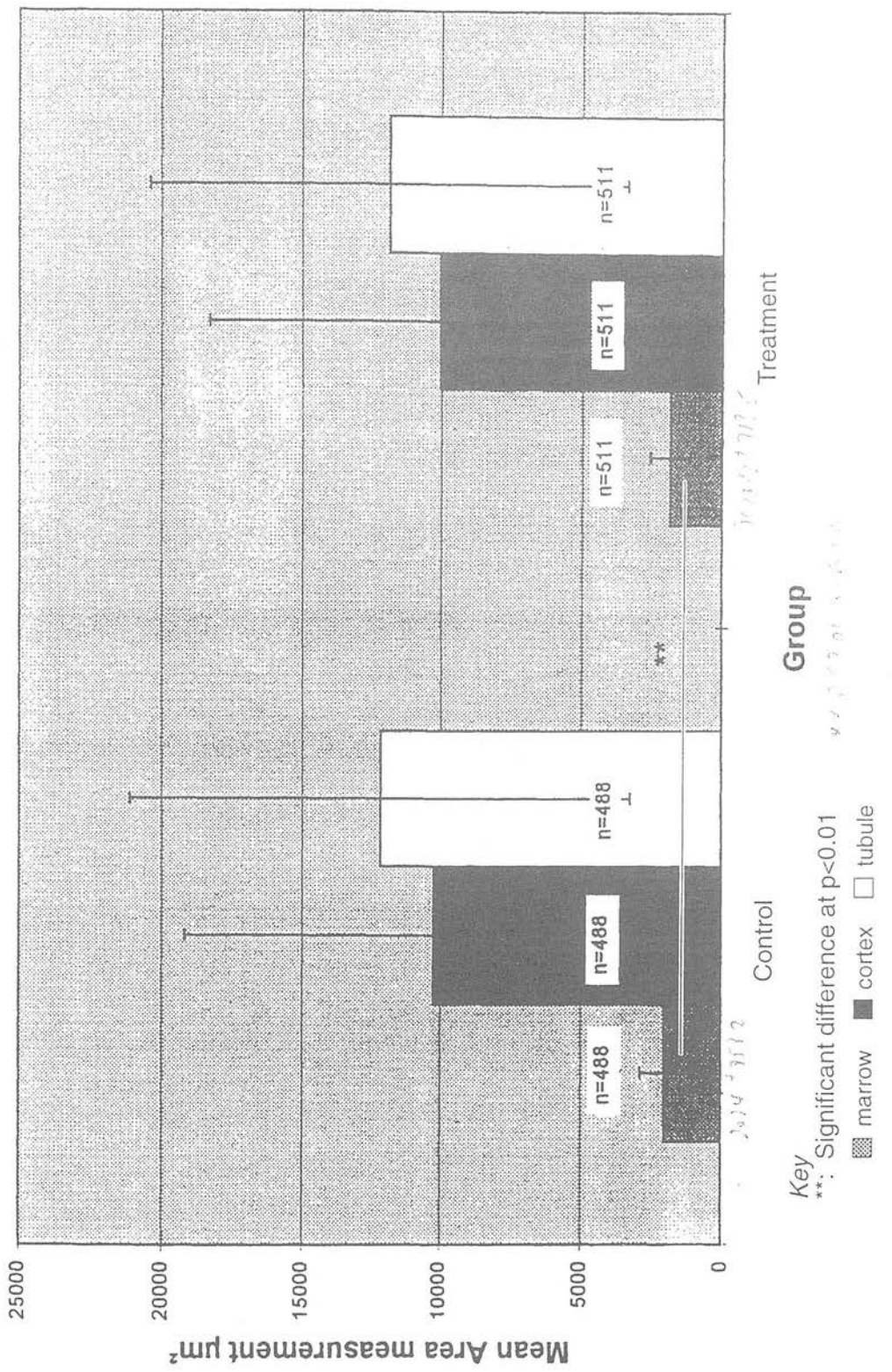


Figure 4.15: Mean (±S.D) group marrow, cortex and tubule area measurements



4.4.4 Comparison of absolute group marrow area measurements by zone

Group marrow analysis by zone was conducted by ANOVA testing on the stacked data with "between zone" comparisons analysed by Tukey testing.

A significant difference was observed ($P<0.01$) between Control and Treatment absolute marrow area means in zone 1 (see Figure 4.16). The Treatment group had a significantly smaller mean marrow size than the Control group in zone 1. The Treatment Zone 1 marrow mean was $1618 \pm 626 \mu m^2$ compared with 2162 ± 930 μm^2 in Control Zone 1.

There was no significant difference between absolute mean marrow measurements in the remaining three zones. The absolute mean values for group marrow, cortex and tubule areas by zone are given in Table 4.6.

4.4.4.1 Between pony comparison of zone 1 mean absolute marrow area measurements

Zone 1 mean marrow measurements were then analysed for differences between horses by ANOVA testing with "between horse" comparisons evaluated by Tukey testing. A significant difference was found ($P<0.05$) between Treatment pony no.1 and Control pony no.1 and also between Treatment pony no.2 and Control pony no.2 (see Figure 4.17). There was no significant difference observed between any other pairs.

The absolute mean values for marrow, cortex and tubule areas by individual are given in Table 4.7.

4.4.4.2 Mean area measurement by zone across the hoof wall (see Table 4.8 below)

Figures 4.18, 4.19 and 4.20 illustrate the mean area measurements for marrows, tubules and cortices respectively, stacked for the entire trial group (ie; Treatment and Control animals), for the four mid zone sampling sites.

The data for Treatment animals 5 and 6 for zone 1 were excluded from the marrow stack because of the individual treatment effect on marrow size described in Section 4.4.4.1 above. Similarly, because cortical mean absolute area was determined by difference from data from the same individuals, these too were excluded from the cortical stack.

Figure 4.16: Mean (±S.D) zonal marrow area measurements for Control and Treatment groups

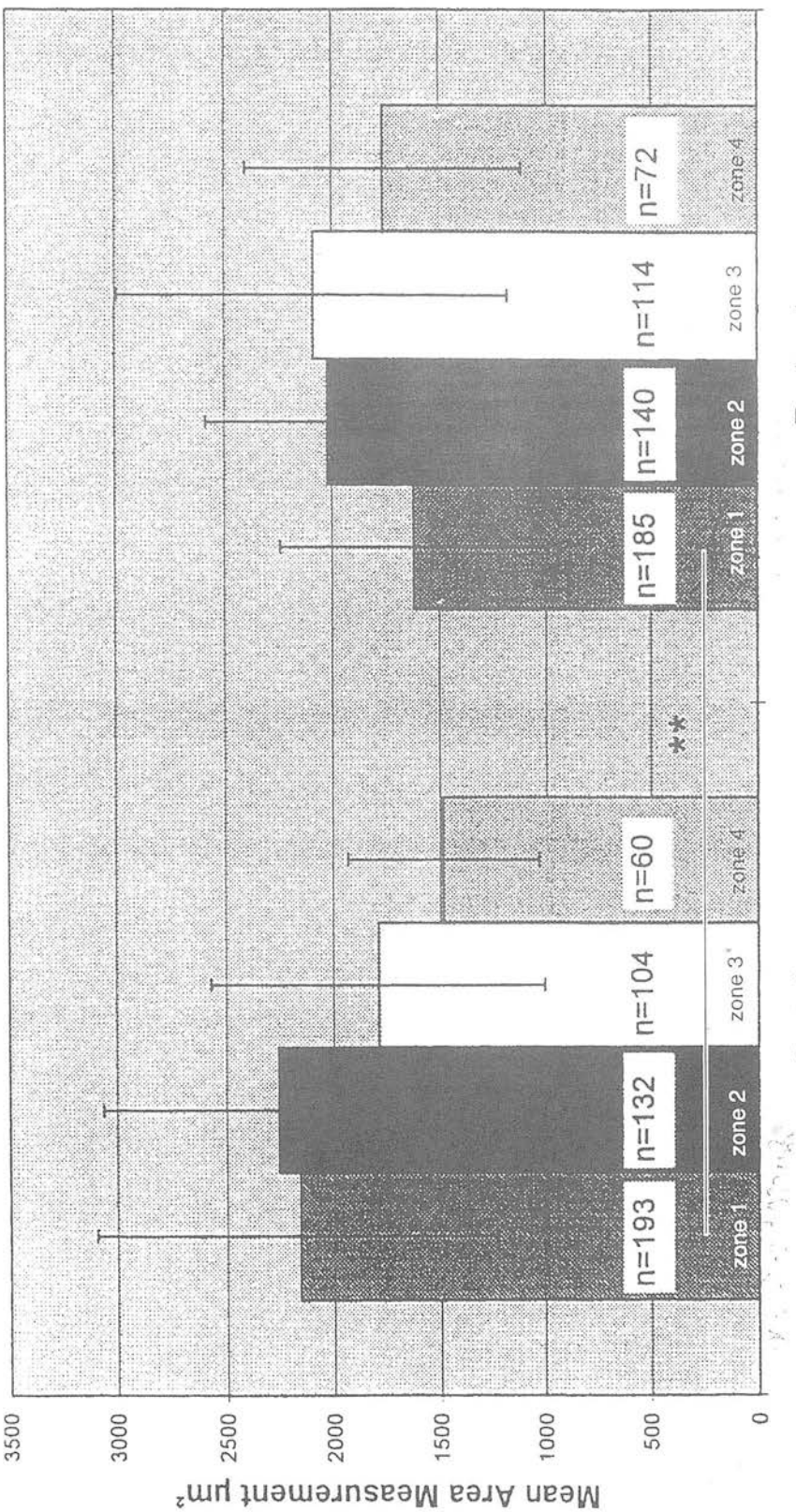


Table 4.6: The absolute mean values for marrow cortex and tubule areas by zone

Group		Absolute area measurement μm^2					
Control Group	n	Marrow	±SD	Cortex	±SD	Tubule	±SD
Zone 1	193	2162	930	7002	4319	9164	4686
Zone 2	132	2259	801	8185	4490	10444	4739
Zone 3	104	1788	780	12073	8475	13395	8427
Zone 4	60	1484	448	22158	15314	23641	15529
Treatment Group							
Zone 1	185	1618	626	7167	5434	8782	5559
Zone 2	140	2020	566	8904	5461	10925	5662
Zone 3	114	2085	904	10620	7991	12705	8395
Zone 4	72	1759	645	18726	12379	20486	12725

Table 4.7: Mean Zonal Absolute Measurements (μm^2) of Marrow, Cortex and Tubule by Individual

Control 1

	C1Ma mean	C1Tu mean	B1Co mean
Zone 1	2598.07	7616.16	5081.13
Zone 2	2622.37	14306.97	11684.60
Zone 3	1993.66	16454.88	14461.22
Zone 4	1631.13	23483.46	21852.33

Control 2

	C2Ma mean	C2Tu mean	B2Co mean
Zone 1	3185.43	14104.55	10919.13
Zone 2	2846.45	12914.79	10068.34
Zone 3	2651.60	16262.67	13611.07
Zone 4	1759.59	27761.21	26001.63

Control 3

	C3Ma mean	C3Tu mean	B3Co mean
Zone 1	1475.21	7282.63	5807.42
Zone 2	1727.97	8019.93	6291.97
Zone 3	1032.29	9748.05	8715.75
Zone 4	1286.51	15038.80	13752.29

Control 4

	C4Ma mean	C4Tu mean	B4Co mean
Zone 1	1793.72	9966.50	8172.77
Zone 2	2165.89	8661.01	6495.11
Zone 3	1930.97	13476.22	11545.22
Zone 4	1249.38	29235.83	27986.44

Treatment 1

	T1Ma mean	T1Tu mean	T1Co mean
Zone 1	1507.77	16236.50	14728.73
Zone 2	1786.33	15089.29	13302.88
Zone 3	2286.73	15008.45	12721.72
Zone 4	2448.98	37222.11	34773.13

Treatment 2

	T2Ma mean	T2Tu mean	T2Co mean
Zone 1	2334.46	10225.57	7900.25
Zone 2	2435.15	13081.56	10646.40
Zone 3	2824.70	17757.38	14932.68
Zone 4	2151.10	21996.31	19845.22

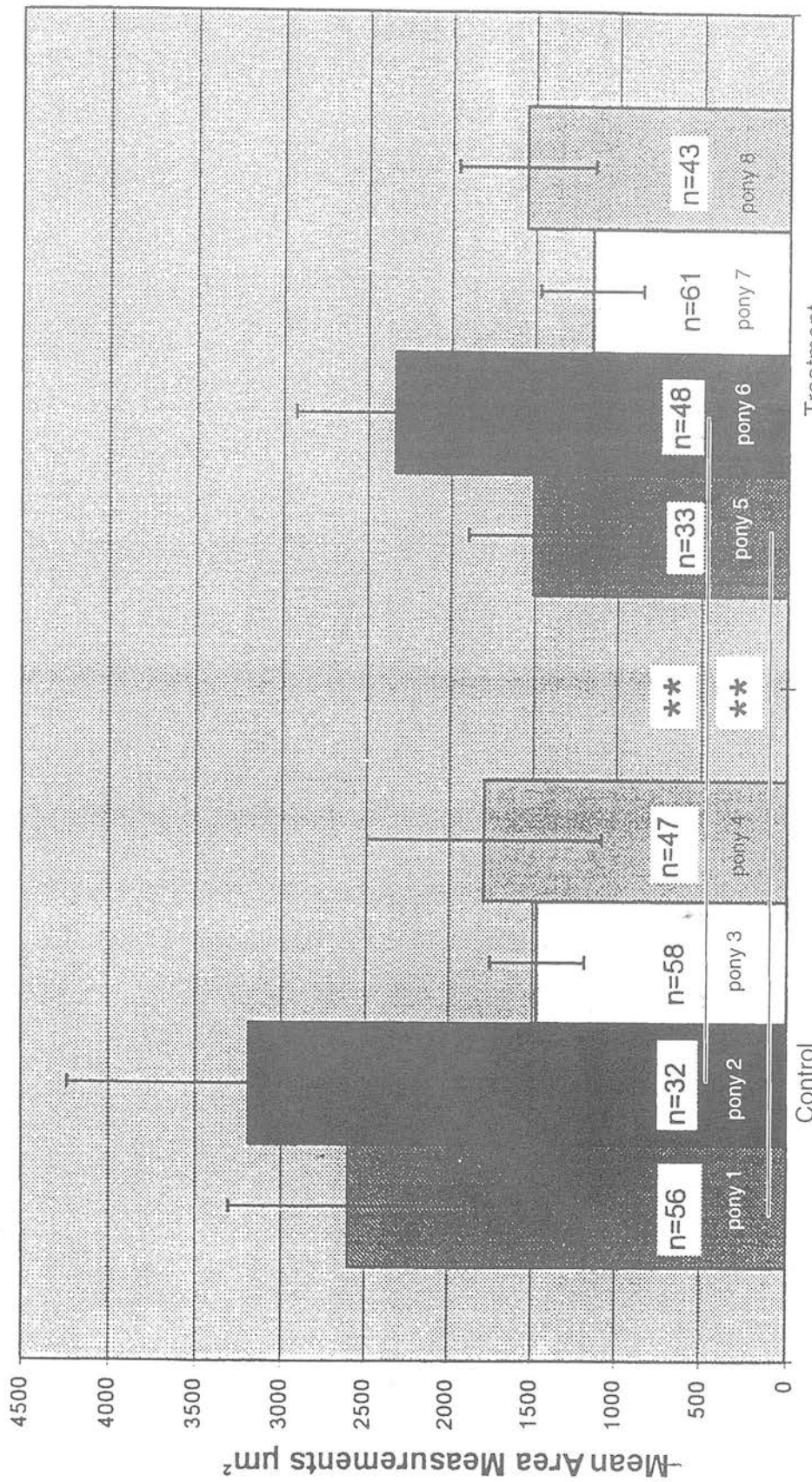
Treatment 3

	T3Ma mean	T3Tu mean	T3Co mean
Zone 1	1159.68	5792.80	4633.13
Zone 2	2077.78	9167.29	7089.51
Zone 3	2305.03	11760.18	9455.14
Zone 4	1578.07	14663.42	13685.35

Treatment 4

	T4Ma mean	T4Tu mean	T4Co mean
Zone 1	1553.39	5704.00	4150.61
Zone 2	1767.51	8462.37	6694.86
Zone 3	1290.79	9162.02	6871.26
Zone 4	1165.80	16425.71	15259.91

Figure 4.17: Mean (±S.D) marrow area measurements in zone 1 by individual pony



Key
**: Significant difference at p<0.01

Figure 4.18: All pony mean (±S.D) marrow area measurements by zone (excluding treatment animals 5 and 6, zone 1 data)

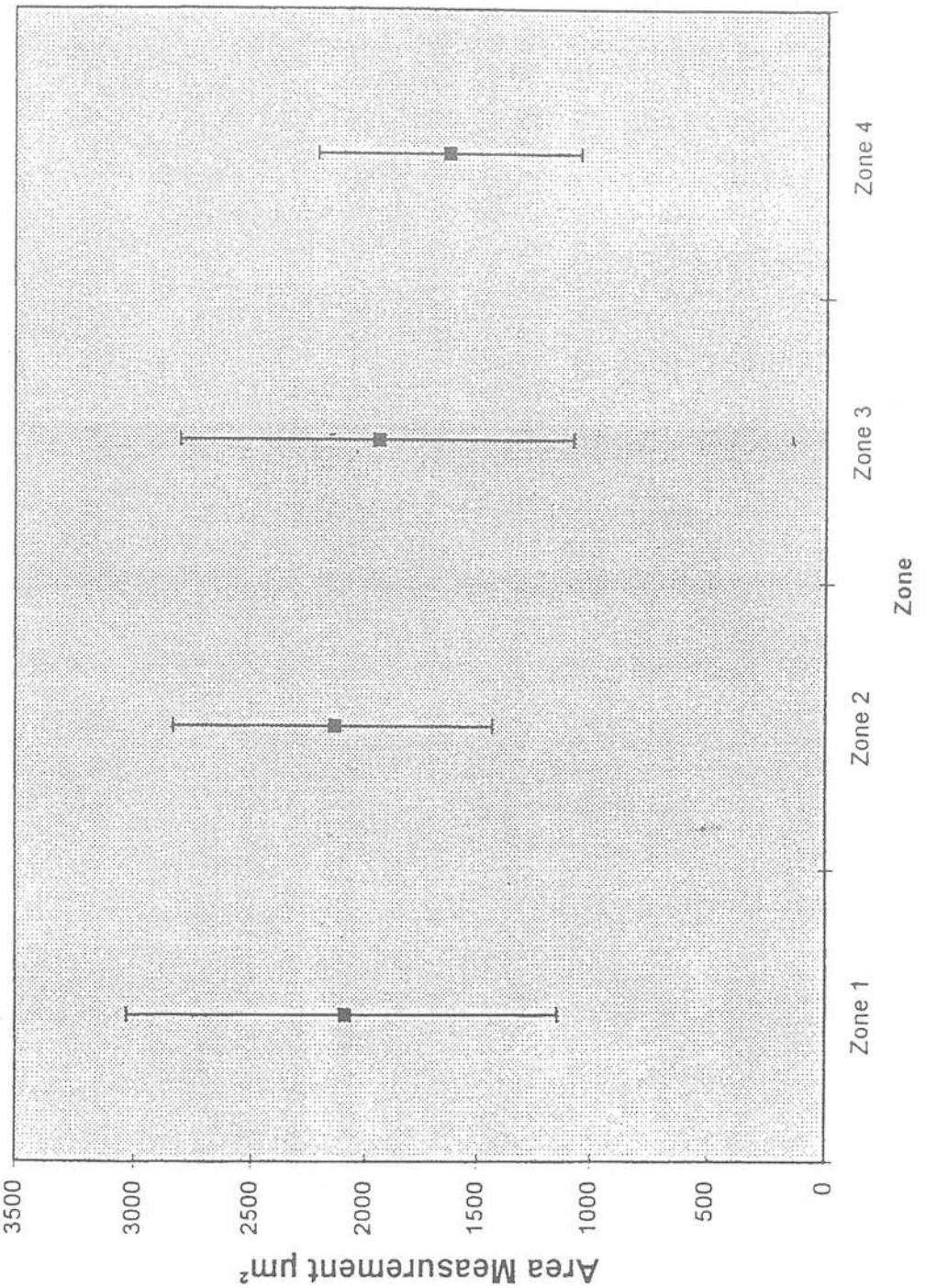


Figure 4.19: All pony mean (±S.D) tubule area measurements by zone (no exclusions)

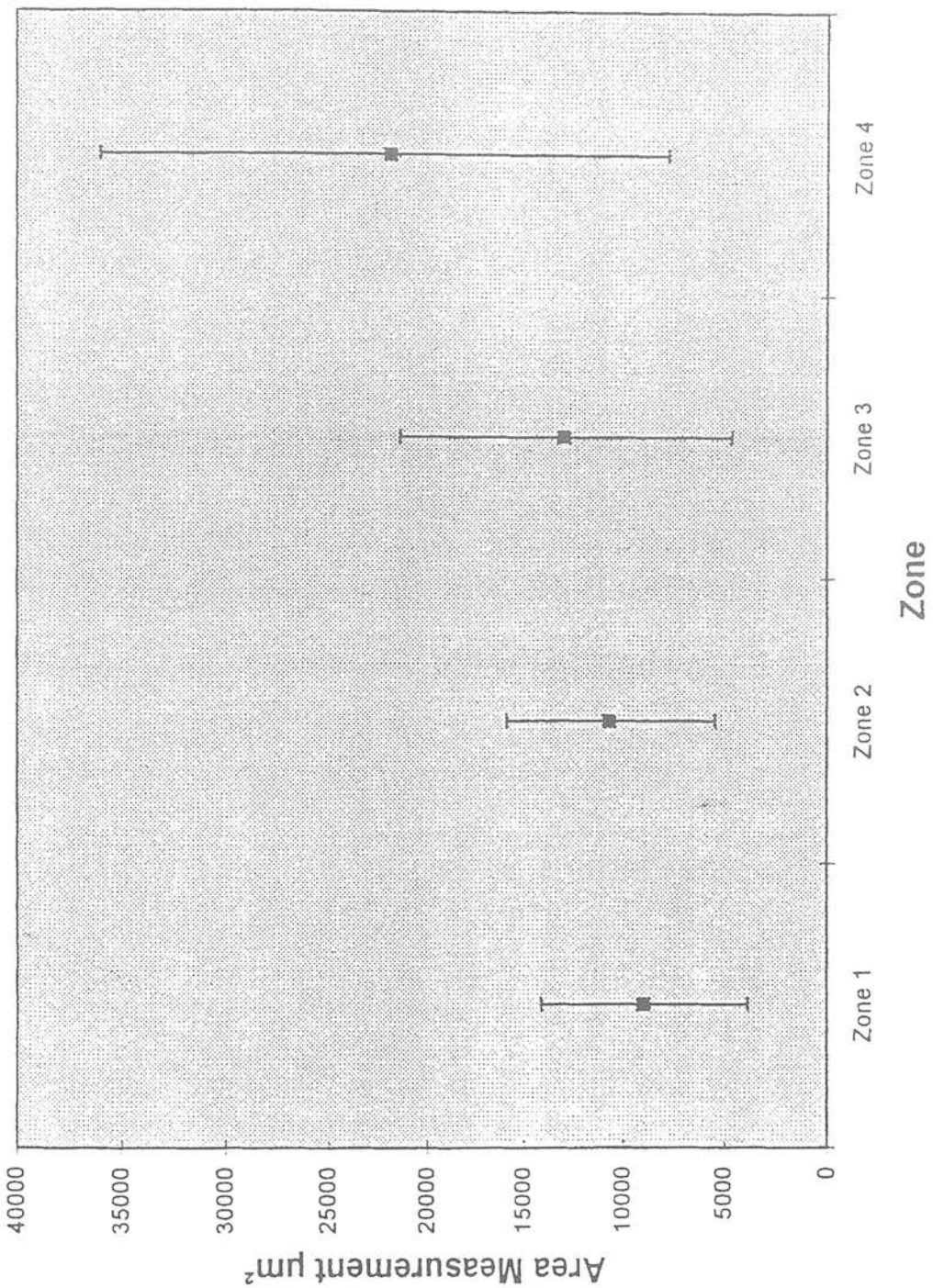
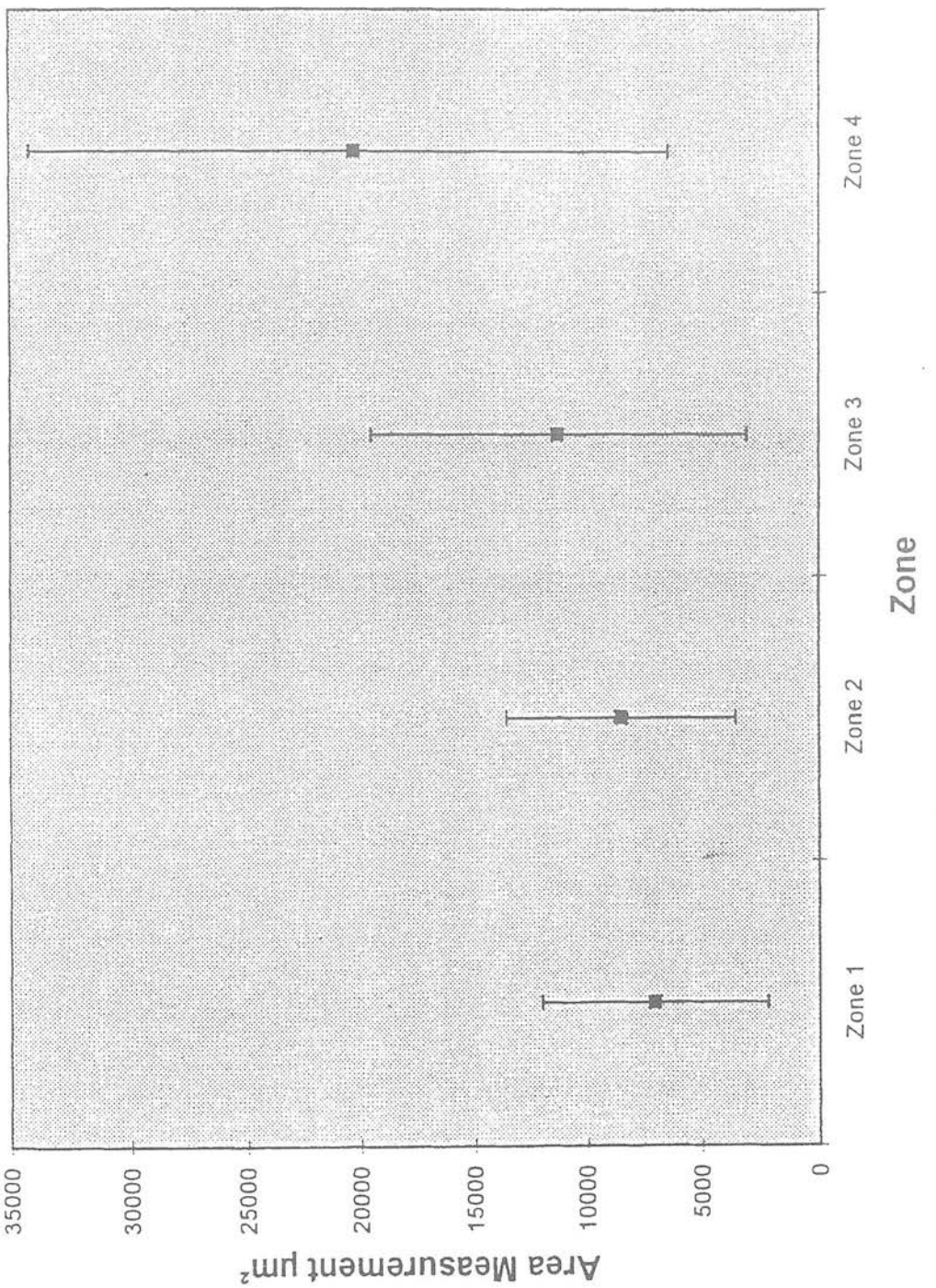


Figure 4.20: All pony mean (±S.D) cortex area measurements by zone (excluding treatment animals 5 and 6, zone 1 data)



In Figure 4.18 there appears to be a broad trend of decreasing marrow mean size with increasing depth across the hoof wall from 2088μm^2 to 1634 μm^2.

The tubule plot (Figure 4.19) illustrates a progressive increase in tubule size from zone 1 to 4. Tubule absolute area measurements in zones 1 to 3 increase gradually from a mean of 8976 μm^2 to 13034 μm^2 and then there appears to be a relatively large but not significantly different increase in the size of zone 4 tubules. The cortical plot (Figure 4.20) follows a similar pattern to that described for the tubule population.

Table 4.8 below gives the mean absolute area measurements for marrow, tubule and cortex for all ponies excluding Treatment 1 and 2, as previously explained.

Table 4.8 Mean absolute area measurements by zone

Mean Absolute Area Measurements	Marrow* ± SD n=297 (μm^2)	Cortex* ± SD n=297 (μm^2)	Tubule ± SD n=379 (μm^2)
Zone 1	2088 ± 982	6103 ± 3921	8976 ± 5129
Zone 2	2136 ± 699	8555 ± 5017	10691 ± 5231
Zone 3	1943 ± 858	11313 ± 8239	13034 ± 8398
Zone 4	1634 ± 578	20286 ± 13843	21920 ± 14101

* Excludes Treatment 1 and 2, Zone 1 because these were found to be significantly different from the rest.

4.4.5 Group zonal area fraction measurements

Group zonal area fraction measurements for marrow cortex and tubule, and intertubular horn by difference, were calculated according to Figure 4.21. The results are summarised in Table 4.9 and are illustrated in the pie chart in Figure 4.22. Zonal area fraction values by individual pony are given in Table 4.10.

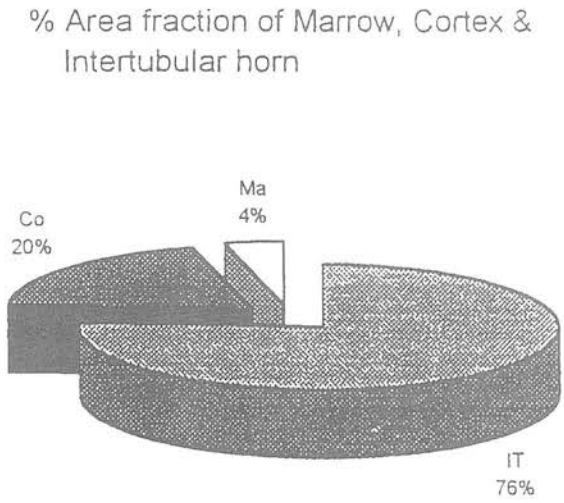
4.4.5.1 Marrow area fractions

Marrow area fraction decreased with increasing depth into the hoof wall. Marrow area fraction values were different in each respective zone (see Table 4.9) ranging from 1.5 - 6.4% in the Control Group compared with 2.0 - 4.9% in the Treatment Group. However, the significant difference found in absolute marrow size between control and treatment marrow size was not reflected in a difference in area fraction by chi square test.

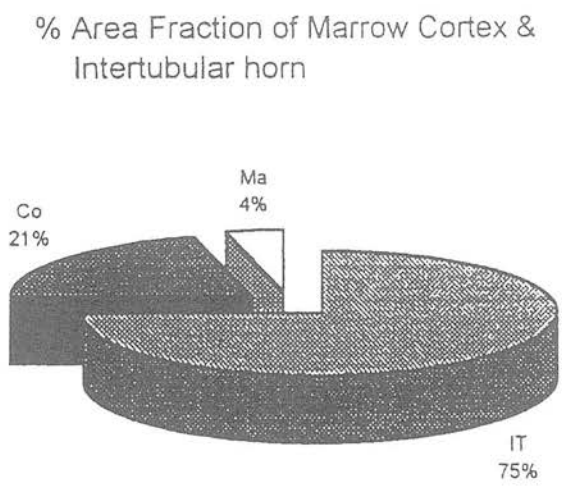
Figure 4.21: Area fraction measurements made in each zonal sampling site

Figure 4.22: Area fraction of cortex, marrow and intertubular horn by group

a) Control



b) Treatment



Key
Ma: marrow
Co: cortex
IT: intertubular horn

Table 4.9: Group comparison of zonal area fractions of marrow, cortex and tubule

	Marrow %		Cortex %		Tubule %	
	Control	Treatment	Control	Treatment	Control	Treatment
Zone 1	6.4	4.9	21.0	22.0	27.4	27.0
Zone 2	4.7	4.4	17.0	20.0	22.0	24.0
Zone 3	3.0	3.8	19.0	20.0	22.0	24.0
Zone 4	1.5	2.0	23.0	22.0	25.0	24.0

Table 4.10: Zonal area fraction of marrow, cortex and tubule by individual (%)

Treatment 1

T1	Ma	Tu	Co	IT
Z1	3.43	36.92	33.49	63.08
Z2	2.91	24.58	21.67	75.41
Z3	3.97	26.08	22.10	73.92
Z4	1.73	26.35	24.61	73.65

Treatment 2

T2	Ma	Tu	Co	IT
Z1	7.19	31.51	23.33	68.49
Z2	4.76	28.56	20.80	74.44
Z3	4.58	28.79	24.21	73.13
Z4	2.63	26.87	24.24	71.21

Treatment 3

T3	Ma	Tu	Co	IT
Z1	4.55	22.74	18.19	77.26
Z2	6.14	27.11	20.97	72.89
Z3	3.70	18.86	15.16	81.14
Z4	2.35	21.87	19.52	78.13

Treatment 4

T4	Ma	Tu	Co	IT
Z1	4.78	17.55	12.77	82.45
Z2	4.48	21.46	16.98	78.54
Z3	3.39	21.46	18.06	78.54
Z4	1.46	20.62	19.16	79.38

Control 1

C1	Ma	Tu	Co	IT
Z1	9.03	24.46	17.34	73.53
Z2	4.24	23.14	18.90	76.86
Z3	2.67	22.02	19.38	77.98
Z4	2.06	29.19	27.16	70.80

Control 2

C2	Ma	Tu	Co	IT
Z1	6.35	28.13	21.78	71.87
Z2	4.96	22.49	17.54	77.50
Z3	3.52	21.56	18.04	78.44
Z4	1.56	24.62	23.06	75.38

Control 3

C3	Ma	Tu	Co	IT
Z1	4.78	26.04	20.76	73.96
Z2	4.48	20.96	16.44	79.04
Z3	3.39	20.48	18.31	79.52
Z4	1.46	13.98	12.78	86.02

Control 4

C4	Ma	Tu	Co	IT
Z1	5.29	29.39	24.10	70.61
Z2	5.17	20.66	15.50	79.34
Z3	3.61	25.20	21.59	74.80
Z4	1.24	28.94	27.71	71.06

4.4.5.2 Tubule area fractions

The pattern of tubule area fractions across the hoof wall contrasted with that recorded for the marrows (see Table 4.9). Zone 1 tubule area fraction was noticeably, although not significantly, higher in zone 1 compared to all other zones.

4.4.5.3 Cortical area fractions

The cortical area fraction pattern (see Table 4.9) reflected the changes seen both in tubule and marrow trends across the wall. The net effect of a decreasing marrow size by depth into the hoof wall is the appearance of what may be a "3" zone pattern of cortical area fraction. The cortical area fraction in zones 1 and 4 were higher than zones 2 and 3.

4.4.6 Tubular: intertubular ratio of area fraction

The pie-chart in Figure 4.22 illustrates the area fraction of tubular (comprising cortical and marrow area fractions) and intertubular horn calculated for the Control and Treatment groups. No significant differences were found between the groups by chi square test. The intertubular horn accounted for an average 75.5% of the hoof wall at the MDC, giving an intertubular to tubular horn ratio of approximately 3 : 1 for this site. This was new anatomical information.

4.5 Discussion

4.5.1 Normal anatomical findings

4.5.1.1 Marrow, cortex and tubule mean size and area fraction

The broad trend towards a decreasing marrow mean size with increasing depth into the hoof wall as seen in Figure 4.8 agrees with preliminary results produced by Kasapi and Gosline (1997). However, in their work, Kasapi and Gosline (1997) took measurements from only two animals and they did not quote standard deviations. Thus, from the results of this work alone, it is difficult to judge whether a decrease in marrow size is a generalized equid functional pattern as individual variation can have a large effect on the results obtained. Nevertheless, a decrease in marrow size by depth into the hoof wall could have interesting functional effects: Leach (1980) suggested that the marrow acts as a 'stress raiser'; that is, it allows the concentration of stress on its outer margin. Thus, the larger mean marrow size in zone 1 may be another mechanism for dealing with stress transfer across the hoof wall in the same way that a

relatively large tubule density in the outer SM may concentrate stress as suggested in Chapter 3.

Since mean tubule and cortical sizes are seen to increase across the hoof wall, in contrast to the change in marrow size, then the combined effects of high TD (from Chapter 3), high marrow size and small tubular size may serve to produce an increased chance of cracking in the outer layers of the SM only, as suggested in Chapter 3. Certainly in terms of area fraction, as shown in Table 4.9, marrow area is seen to decrease from approximately 6% in zone 1 of the SM to only 1.5% in zone 4. These results for area fraction are certainly in agreement with those of Kasapi and Gosline (1997) who recorded a marrow area fraction of 4.5% in the outer hoof wall and 1.5% in the inner hoof wall.

Tubule, and similarly cortex, sizes however are seen to increase with depth into the hoof wall. This may imply that the tubule and cortex have different functions to the marrow, and their area fractions can be seen to be concentrated in the outer and inner zones of the SM (Table 4.9). The larger TD in zone 1 of the SM accounts for the relatively high volume fraction of cortex found here, whereas in zone 4, fewer, larger tubules account for the volume fraction of cortex found at that site. It can be speculated that these histomorphmetric differences result in differences in stress transfer across the wall, but until measurements of mechanical properties have been examined in Chapter 5, any inter-relationships between these parameters cannot be determined.

4.5.1.2 Intertubular: tubular horn area fraction ratio

The morphometric analysis of the total tubular and intertubular horn area of the hoof wall has established an intertubular to tubular horn area ratio of 3 : 1. This differs from the measured value stated for Warmbloods of 3 : 2 by Bucher (1987) and the generally assumed value of 1 : 1 quoted by Bertram and Gosline (1986), Thomason *et al* (1992) and Douglas (1993). The precise method adopted by Bucher (1987) for measuring this ratio is unclear. It is also unknown whether the values reported by other authors refer to the whole wall or from a specific zone within the wall. As the values for the material properties of tubular compared with intertubular horn are not known, the implications of this finding in terms of stress transfer across the hoof wall

are not deducible. The contribution of tubular and intertubular horn to mechanical properties needs to be determined. Chapter 6 considers whether correlations exist between the mechanical properties investigated in Chapter 5 and morphometric features. A similar analysis of Warmblood hoof horn using the methods used in this study is required to clarify the difference in intertubular: tubular horn ratio found in this work and that found by Bucher (1987).

4.5.2 Treatment (biotin)-induced differences

The significant decrease in mean marrow size in the treatment group compared with the controls suggests that biotin supplementation may affect horn production from the apical tip region of the dermal papillae. An increase in papilla tip size, or in effective tip size in terms of cell production from it, may be occurring with biotin supplementation. A comparison of the distribution of absolute area fraction measurements reveals a marked shift in marrow population towards a smaller marrow size (see Figure 4.14). This trend is clearly seen at the level of the group but is significantly more pronounced within zone 1, see Figure 4.16, and within certain individual horse pairs; pony 1 compared to pony 5 in particular and in pony 2 compared to pony 6 to a lesser extent (See Figure 4.17). This indicates that the treatment effect is both zone specific and could also be age related.

Marrow size may decrease by a reduction in the amount of degenerative change at its border. This theory was favoured by Geyer *et al* (1988) when they observed the phenomenon of decreasing marrow size with biotin supplementation in one horse. An alternative explanation is that the papilla tip has simply become more productive along its length. Either way the "effective size" of the horn-producing tip of the papilla has been increased. The *specific* mechanism by which the "effective size" of the horn-generating site at the papillae tip is increased in response to supplementation is not known. Whether this may occur as a result of changes in the size or shape of the papillae tip, or an extension of the germanitive layer towards its apex, or by the mitotic activation of previously dormant keratinocytes, are all questions that need to be investigated by conducting experiments or measurements at the cellular level and will require a stereological approach.

Some form of limitation to horn production may exist along the longitudinal axis of the papillae in the control group, or alternatively a response to super supplementation has occurred in the treatment group. Answers to these questions may come by investigating biochemical and cellular volume and turnover changes at the cellular level in supplementation trials with varying biotin levels.

The zone-related response in treatment animals compared with control animals may indicate that zonation occurs for dermal papillae with different types of papillae present within the coronary corium or that nutritive supply to them may differ according to location within the corium. It also verifies the conclusion that zonation of the hoof wall occurs, as has been seen with tubule density in Chapter 3.

Geyer and Tagwerker (1986) described changes in marrow sizes in response to biotin deficiency in pigs. These changes were likewise restricted to the outer portion of the horn. In addition, the decrease in marrow size seen in this work may account for the observed decrease in marrow area fraction reported by Geyer *et al* (1988) for a single horse. However, Geyer *et al* (1988) reported a different absolute marrow area reduction but the overall reduction in marrow area from 2.0% to 1.5% is in the same order as marrow area fraction found in this work. Pollitt (1990) described differences in papillae length across the coronary band width in horses. In cattle, where tubule zonation has also been subjectively described (Wilkens 1955, 1964), considerable variation in the shape of the papillae has been documented (Schummer *et al* 1981). Papillae shape and size differences have not been measured objectively in this work because the work concentrated on epidermis.

The age-dependent effect observed in this study is consistent with the findings reported in pigs (Triebel and Lobsiger 1979) and horses (Dittrich *et al* 1994). This dependency may indicate that dermal papillae and tubule marrow formation undergo a maturation process and supplementation may have a particular age 'window' to effect changes. Future trials should seek to investigate this possible age related response to biotin supplementation.

4.6 Conclusions

There was an effect of biotin supplementation on tubule marrow sizes: Treatment group animals had a mean tubule marrow size of 1852 ± 713.5 μm^2 and

control group animals had a mean marrow size of 2024 ± 858.2 μm^2 ($p<0.01$ by ANOVA). This difference was found to be accounted for by a difference between the two younger pony pairs and the differences were found in Zone 1 of the SM only. The tubular to intertubular horn area fraction ratio which was 1:3 in this work and not 1:1 as has been estimated by other workers in the past.

CHAPTER 5

The mechanical properties of pony hoof wall at the MDC

5.1 Introduction

This chapter investigates the micro mechanical and moisture properties of the hoof wall from the ponies in this trial. The functional results obtained from material properties testing methods in this chapter can then be related to the other biological data obtained elsewhere in the thesis, and an assessment of the biotin effects on these properties can be made.

5.1.1 Stress-strain relationships

The mechanical behaviour of a structure such as the hoof wall reflects a relationship between an applied load, also known as a force or a **stress**, and its response to that stress which is its deformation or **strain** (Uvarov *et al* 1971).

Figure 5.1 illustrates a typical stress-strain curve which illustrates the nature of deformation (strain) to increasing load (stress).

The initial part of the deformation curve reveals a linear or proportional relationship in which strain is directly proportional to the applied stress. This relationship, over the proportional zone of the stress-strain curve, is referred to as Hooke's law of linear elasticity (after Robert Hook 1635-1703). However, a point is reached, known as the **proportional limit** or **elastic limit** (Mohsenin 1970) at which a departure from stress-strain linearity occurs.

The fundamental relationship between stress and strain for any given material is derived from the character of the forces acting at a molecular level within a material (Gordon 1976). In the unstressed state an equilibrium position exists in which the interatomic distance is such that interatomic forces are balanced, reflecting the lowest energy state for the material. The application of an applied force alters interatomic distances which increases interatomic forces which raises the energy level within the material (Gordon 1976).

Figure 5.1 The form of a stress-strain graph for a Hookean Material

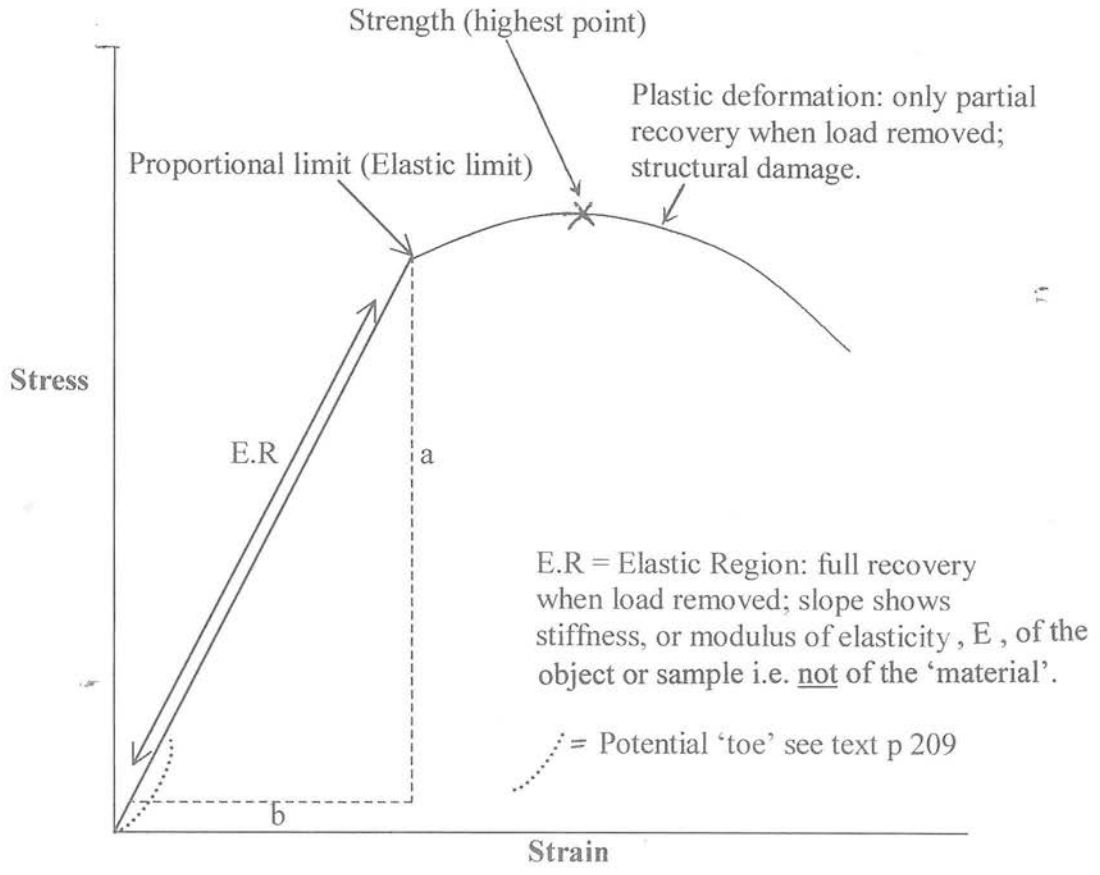


Figure 5.1 Information:

The **yield point** is the point on the stress-strain graph where the plot becomes appreciably non-linear and represents the greatest stress a material is capable of sustaining without loss of elasticity, or the ability to recover strain completely and instantaneously, when the stress is removed. It is the point beyond which Hooke's Law is not obeyed. The stress level at this point defines the material's **yield strength** or **bioyield** (Callister 1994, Vincent 1992). After the yield strength has been reached, permanent, non-recoverable, **plastic** deformation of the material occurs. This represents the point at which the interatomic bonds can no longer resist the applied stress.

The **slope** of stress strain curve, within the proportional limit is defined as the **modulus** or, **stiffness**, E.

This can be calculated from a/b above or from the slope of a least squares fit through the stress-strain data.

The **slope** of the stress-strain curve in the proportional zone is defined by a constant: Young's **modulus of elasticity** (after Robert Young 1773-1829). This **modulus, 'E',** is a measure of the **stiffness** of a given material (Wainwright *et al* 1976). The greater the modulus the stiffer the material i.e. the smaller the strain or deformation at any given stress. The relationship between stress and strain can thus be expressed as:

Stress = modulus of elasticity x strain

An understanding of how a material responds to externally applied forces is an essential part of interpreting microscopic and macroscopic hoof function and dysfunction. When a solid object is subject to an external force the object deforms in accordance with Newton's third law of motion in order to oppose the force. The size of the deformation, for a given material under defined conditions, is dependent upon the magnitude of the applied force and the dimensions of the object.

Stress is defined as a measure of the applied force per unit area over which the force is applied:

$$\text{Stress} = \frac{\text{Force}}{\text{Area}}$$

Stress has units of force per unit area (or pressure). The SI unit of stress is the Newton per square metre, or Pascal (named after Blaise Pascal (1623-62)).

Strain is a measurement of the resultant deformation in the direction of the applied stress normalised to the original length of the object:

$$\text{Strain} = \frac{\text{change in length of sample}}{\text{original length of sample}}$$

Since strain is a ratio of change in dimension per unit of dimension, it is a unitless number.

Thus, since modulus or **stiffness**, **E**, $= \frac{\text{stress}}{\text{strain}}$ and strain is unitless, then **E** has units of Newtons per square metre or Pascals.

5.1.2 Response of hoof horn structure to loading (compression, tension and bending)

The response of a structure to loading will be a function of the geometric form of the structure and the intrinsic proportions and properties of its constituent materials, (Vogel 1989).

Vincent (1992) points out that it is important to decide whether it is a 'material' or a 'structure' that is under test in an investigation of mechanical properties. From Figure 1.6 and from Chapter 1, this investigation could take place at many different levels of the structural hierarchy of hoof horn. In the case of this thesis, the 'material' under investigation is the tubular and intertubular epithelial horn of the *stratum medium* of the hoof wall, whereas the 'structure' under investigation is the hoof wall at the MDC.

The stress-strain relationship within hoof horn is dependent upon its material properties. However, the response to loading of a structure is also dependent upon the arrangement of its constituent material (Vogel 1989). The mechanical properties of a material reflect both the material present and its spatial arrangement (Vogel 1989).

The implication from this is that the morphology of the structure being investigated is at least as important as its mechanical properties; the science of mechanical tests on biological materials is an extension of morphology and follows directly from the work of the great anatomists of the last century (Vincent 1992). In this way, mechanical testing of hoof horn is a logical sequel to an investigation of its morphometric properties.

The absolute measurements for components and the proportions of constituents of the hoof wall at the MDC have been investigated in Chapter 4. This, together with the TD results from Chapter 3, have established an objective data set for some of the anatomical properties of the hoof wall. This chapter describes mechanical testing that was carried out in order to determine the material properties of the hoof wall at the same site.

Ideally, the material properties for any given structure should be determined by experiments which replicate the conditions under which it performs. Past

approaches to determining the mechanical properties of hoof, from the literature, have included hardness testing (Brooks and Simmins 1980, Webb, Penny and Johnston 1984, Simmins and Brooks 1985, Reilly and Brooks 1990), compression testing (Butler and Hintz 1977, Leach and Zoerb 1983, Landeau *et al* 1983, Kasapi and Gosline 1996), and tensile testing (Bertram and Gosline 1987, Kung 1991, Zenker *et al* 1995, Douglas *et al* 1996, Kasapi and Gosline 1997).

Tensile testing has often taken the samples past the elastic limit to the point of destruction, or ultimate strength, which has resulted in fracture. Different results have been obtained from such studies (see Table 5.1). Each result reflects the particular circumstances of the testing conditions and, because many of them are different they are difficult to compare directly, as many factors can influence the fundamental relationship between stress and strain that is being tested. These include the mode of stressing the sample (eg: in tension compression or bending), the site within the hoof capsule from which the sample was taken (Leach 1980), the geometry of the sample (Jackson 1992), the moisture content (MC%) of the sample (Bertram and Gosline 1987) and the speed of testing (Jackson 1992). As Douglas *et al* (1996) point out, previous studies have not controlled for these factors.

Whilst tensile and compressive stresses occur within the hoof during loading, it is generally rare that structures experience only pure tension or pure compression alone. More often structures are also bent or twisted in combination with pure tensile and/or compressive loading (Wainwright *et al* 1976, Biewener 1992).

Section 1.6 of the literature review, and the conclusions of Lungwitz (1891), supported by the findings of Thomason *et al* 1992 and Douglas *et al* (1996) indicate that the principle forms of deformation experienced by the hoof capsule are bending and compression. Hood *et al* (1992), used tranducers capable of discriminating between bending and compressive deformation, and observed that the dorsal hoof wall was subject to either pure bending, or compression and bending, during static weight-bearing. Pure compression within the wall was not recorded.

From this it was concluded that the most important functional mechanical property to measure for the hoof wall was its **stiffness** or **modulus** below the elastic limit, and that this would be done in **bending** tests.

Table 5.1 Horn stiffness results from previous authors

Moisture status	Area of Hoof Horn Tested	Mechanical Test	E(MPa)	Similar Test to this work conditions	Crosshead Speed mm/min^{-1}
Fresh					
Leach 1980	Outer wall	Vertical CT	3.6	No	1.3
Butler and Hintz 1977	"mid toe region"	CT	3.7	No	1.3
Kung 1991	Coronary horn	Tensile	4.5	No	Not given
Zenker *et al* 1995	Coronary horn dorsal wall	Tensile	46.0	No	Not given
Webb *et al* 1984	Pigs	CT	9.3	No	1.6
Zoerb and Leach 1978	Various samples	CT	215.0	Yes	0.5
Leach and Zoerb 1983	Inner wall (near MDC)	Vertical CT	237.4	Yes	1.3
Landeau *et al* 1983	SM	CT	240.0–480.0	Yes	1.3
Leach and Zoerb 1983	Outer wall	Vertical CT	354.9	Yes	1.3
Douglas *et al* 1996	Dorsal outer wall Dorsal inner wall Dorsal outer wall Dorsal inner wall	Tension Tension Compression Compression	955.0 502.0 1004.0 523.0	No No No No	5.0 5.0 5.0 5.0
Kitchener and Vincent 1987	Gemsbok horn shaft	3 point bending	4300.0	No	2.0
Hydrated					
Butler 1976		CT	4.3	No	1.3
Betram and Gosline 1987	MDC of SM	100% MC	410.0	No	5.0
Kitchener and Vincent 1987	Gemsbok horn shaft	3 point bending	1800.0	No	2.0
Kasapi and Gosline 1996	MDC toe (parallel to tubules) Various Various	3 point bending CT Tensile	Approx 120.0-400.0 380.0-760.0 280.0-850.0	Partially No No	Various Various Various
Kasapi and Gosline 1997	Inner Middle Outer wall	Tensile	300.0 430.0 560.0	No	1.3
Dry					
Kitchener and Vincent 1987	Gemsbok horn shaft	3 point bending	6100.0	No	2.0
Betram and Gosline 1987	MDC of SM	Tensile	14600.0	No	5.0

5.1.3 Hoof horn bending tests

Since Hood *et al* (1992) concluded that **bending** was the most important physiological aspect of hoof wall biomechanics, it was decided to use a **three point bending technique** (Jackson 1992) to mimic physiological bending within the pony hoof capsule, in order to assess the material properties of pony hoof horn.

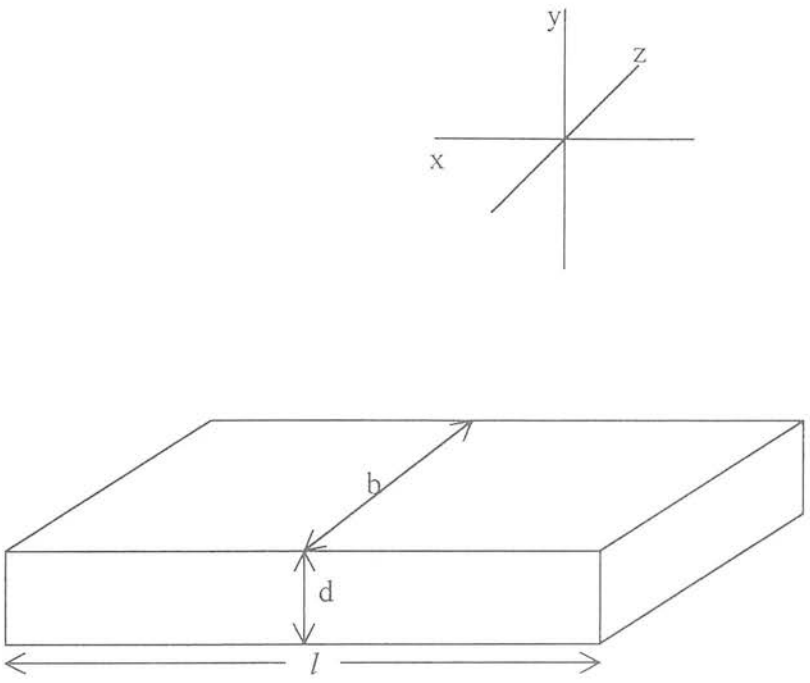
Three point bending of horn relies on cutting small blocks of known dimensions from the hoof wall. These were known as mini **'beams'** of horn which were cut from the MDC. The convention used for referring to the dimensions of these beams, and their orientation within the hoof wall is given in Figure 5.2. The simple theory of beam bending is given in Section 5.1.4.

Three point bending was thought to be an appropriate way to mimic the physiologic movement of the hoof capsule in order to give a more realistic representation of what is happening in the hoof capsule *in vivo*. This was a novel approach, as bending methods had not been used before in any attempts to assess hoof horn mechanical properties. Recently, there has been a move away from destructive testing of biological samples as these tests do not represent reality and their results are often unreliable (Vincent, J. personal communication).

Kitchener (1987) and Kitchener and Vincent (1987) used a three point bending technique to evaluate the stiffness of Gemsbok head horn. The technique was also reported by Waters (1980) to have been used to examine other biological materials such as enamel and dentine. Thus, 3 point bending as a standardised engineering method, is a reliable form of non-destructive testing for the assessment of material properties which has been used to test other biological specimens in the past.

Standard procedures exist for the 'three point' bending method. Using the American Standard Test Method (ASTM E855, 1994), a beam rests on two supports and is stressed centrally (see Figure 5.3). The 'three points' referred to in '3 point bending' are therefore the two support ends and the centre stressing point. As a result of the bending displacement, the face of the beam furthest away from the load is put under tension (that is, the forces acting upon it act *away* from each other), and the face adjacent to the central stressing point is put under compression (that is, the forces acting upon it act *towards* each other), (see Figure 5.3).

Figure 5.2 Dimensions of cut beams

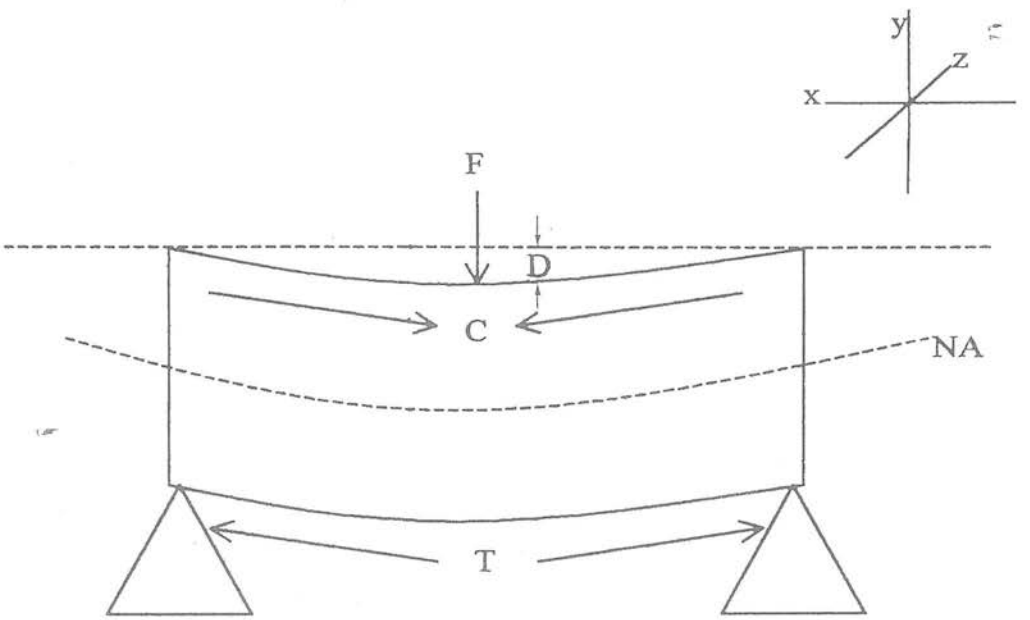


l = length or span of beam, S, (24mm)

d = depth of beam (2mm)

b = breadth of beam = HWD

Figure 5.3: Beam subjected to a bending moment (not to scale)



Key

F = Force
D = Deflection
Δ = Supports
C = Compression
T = Tension
NA = Neutral axis

Hood *et al* (1992) also found that as well as bending being the principle mode of movement of the hoof capsule, it only deformed (strained) through distances of approximately one millimetre *in vivo*. Thus the range of movement through which the test pieces were taken for the work presented in this thesis was also in the order of 1mm. In addition, the beams of horn were known to be in their elastic range during bending (see results section 5.4). Thus, measurements were only taken for the assessment of mechanical properties from experiments that were designed to mimic physiologic movements of representative pieces of horn. They were not taken beyond the yield point or to destruction because Hood *et al* (1992) have shown that the latter tests are unrealistic and inappropriate for 'normal' and healthy hoof samples.

5.1.4 Simple beam theory

The stiffness value of the *material* that makes up a beam is calculated from the stiffness value given by the whole *beam* in bending (the "apparent beam stiffness") and then normalizing the value given for the size of that beam. This needs to be done because the dimensions of a beam will contribute to its apparent stiffness. i.e. a wide beam will have a higher apparent beam stiffness than a narrow beam when made of the same material.

This section explains the theory of how a material stiffness can be derived from an apparent beam stiffness, and the derivations of some of the equations used in achieving its calculation.

In order to interpret the effect of bending moment upon a beam, a number of assumptions need to be made:-

- the material obey's Hooke's law and the proportional limit is not exceeded;
- the beam is of uniform dimensions in the pre-loaded state;
- sections perpendicular to the beam axis remain perpendicular;
- every layer in the beam is free to expand/contract in all directions;
- twisting about the bending plane does not occur.

(Callister 1994).

Figure 5.2 illustrates a beam of horn cut from the MDC to show the dimensions of it which are subsequently used in mechanical calculations:

Figure 5.3 illustrates the forces on the beam in 3 point bending. The deflection of the beam as a result of the bend causes the upper concave section of the beam to be subjected to compression whilst the lower section is under tension. The beam deflects (bends) in accordance with Newton's third law such that an equal and opposite reactive force generates a moment of resistance which opposes the force that has produced the bending moment.

It follows that, at a certain level, a plane exists within the beam that is neither in compression nor tension (Wainwright *et al* 1976). This plane is referred to as the **neutral axis.** Wainwright *et al* (1976) have demonstrated that the stress in the material is proportional to the distance from the neutral axis, but the maximum bending moment occurs at the centre of the beam and so stiffness, **E** is given for the centre of the beam, with maximum **strain** at the top and bottom surfaces of the beam (Bieweiner 1992).

The flexural stiffness or modulus of the beam is given by the product of Young's Modulus for the centre of the beam, which is derived from the slope of the stress-strain curve for the beam, and a function of the distribution of material in the beam about the neutral axis. The effect of this distribution of material is accounted for by the **moment of inertia** or **second moment of area, I.** The second moment of area is a measure of the way material is distributed about a given axis (Wainwright *et al* 1976). **I** represents a measure of the placement of material in a given cross section in the beam with respect to the neutral axis.

$$I = \frac{bd^3}{12}$$ (Jackson 1992)
(Figure 5.2)

Thus, a value for the second moment of area can be calculated from the dimensions of the beam.

Knowing **I**, then a value for the material stiffness ($E_{material}$), as opposed to the beam stiffness (E_{beam}) can also then be calculated.

$$\mathbf{E}_{material} = \frac{E_{beam} \times S^3}{48I}$$

where: S = span of the beam (24mm) ($\equiv l$ in Figure 5.2)

and I = second moment of area (Jackson 1992)

In this way, a **material** stiffness for hoof horn from the MDC can be derived from the beam stiffness that has been given by 3 point bending samples that represent the structure of the hoof wall at the MDC (for a worked example of the calculation of material stiffness from apparent beam stiffness see section 5.4.1).

A **span: depth ratio**, known as an **aspect ratio (A.R.)**, of 10:1, or above, is recommended in bending studies. This is because bending is then concentrated in the centre of the beam with minimum aberrant or artefactual effects which would otherwise be created at the point of bending due to the constrained ends of the beam, were the A.R.<10:1.

5.1.5 Effect of micro moisture content on micro mechanical properties

Section 1.6.4. gave details of the gross MC% of the hoof capsule, the way moisture is grossly distributed within the wall in terms of vertical and horizontal moisture gradients, and the way in which increasing moisture content can decrease the material stiffness of hoof through its effects on increasing inter-molecular bond length. The latter allows increased flexibility to develop within the structure for any given load. For these reasons and those given in section 1.6.4. it is important to control for moisture content when hoof horn samples are analysed for mechanical properties.

Table 5.1 shows that experiments have been designed to ascertain moisture levels within different areas of the hoof wall. However, methods used to calculate hoof moisture content have varied and descriptions of sites of sampling are unclear. The percentage moisture content by weight (gravimetric moisture content) is often cited (Miyaki *et al* 1974, Leach 1980 and Kitchener 1987), but the use of different methods to calculate moisture content can still be confusing.

Jackson (1992) advocates the use of three different equilibrium states to assess MC% and MC% effects on bending properties. These are: firstly, equilibrated at ambient conditions (termed 'fresh' in this thesis); saturated (termed 100MC% in this thesis) and finally; dessicated (termed 'dry' in this thesis).

The stiffness of hoof horn can be highly modified by its MC% according to Leach (1980), Kitchener (1987), Bertram and Gosline (1987). Thus its moisture content, and how it was calculated, must be known and any bending studies must be controlled for MC%.

Until relatively recently surprisingly little heed has been taken of controlling hoof samples for MC% prior to mechanical testing. Thus results obtained in previous studies of the mechanical properties of hoof horn have often reflected the MC% of the samples rather than the inherent stiffness of the material (other than water) that makes up the sample.

Kitchener (1987) and Kitchener and Vincent (1987) examined the effect of water on the stiffness of Gemsbok head horn by using a 3 point bending technique and by varying hydration levels. The water content of the fresh head horn was found to be 20% by weight. The samples were then placed in water for 3 days, the time taken for them to equilibrate (100MC%), at which point they were calculated to have a 40% water content by weight. The samples were mechanically retested at this MC%. They were then dehydrated in an oven at 110°C for 24hrs to produce dry horn (0% MC) and again retested on a 3 point bending apparatus.

The bending tests performed by Kitchener and Vincent (1987) were at a load rate of 2mm/minute and the following results were obtained:

Status of horn sample	Mean stiffness (GPa)
'Fresh' horn	4.3
'Wet' horn	1.8
'Dry' horn (oven dried at 110deg C for 24hrs)	6.1

These results confirmed those of previous studies (Leach 1980; Bertram & Gosline 1987), where the stiffness MC% of horn is inversely related to its MC%.

In order to retrospectively calculate the MC% of samples with respect to their 'dry' (dehydrated) 'wet' (fully hydrated) or 'fresh weights', many tests involve the use of oven drying (Bertram & Gosline 1987) or vacuum drying (Leach 1980). The loss of mass in these instances probably represents a loss of very tightly-bound water or possibly some other volatile component of the tissue (Bertram & Gosline 1987).

In this thesis heating and chemical methods were rejected as possible means of driving moisture from hoof horn beams because thermal and chemical effects may

have affected the properties of the keratin proteins within the samples, which may have altered the characteristics of the beams before mechanical testing. Kitchener (1987) had used a method of equilibrating head horn samples in distilled water for 7 days prior to mechanical testing. This was one method used for controlling moisture status between samples prior to mechanical testing in this thesis. It was decided to determine stiffness at three different moisture contents: 'fresh', 'wet' and 'dry'.

5.2 Aims of this chapter

The aims of this chapter were to determine the bending stiffness of pony hoof horn at a precise MDC location by a three point bending technique at three different moisture contents, and to assess whether biotin supplementation had any effect on moisture content or bending stiffness. The material properties of the hoof wall needed to be established in order to:

i) Assess normality.

ii) Assess whether biotin affects the material properties of hoof horn.

5.3 MATERIALS AND METHODS

5.3.1 Beam sample preparation

Section 3.3.1 of this thesis has described the preparation of MDC blocks from the hoof wall. Figure 3.4 is a flow diagram to show how samples were then cut from the block for histology, TD counting, histomorphometry, and bending.

The blocks had been cling-film wrapped and fridge stored at 4°C for 2 days before they were taken to the mechanical workshop and allowed to equilibrate to air temperature over 2 hrs before further preparation. Three hoof horn beams were then cut from the proximal end of each hoof wall block in consecutive proximo-distal directions using an oscillating saw. They were labelled 'cut 1', 'cut 2', and 'cut 3' respectively. The way in which the beams were cut and distances to the point of cutting were calculated, with respect to the DLNHG between pairs, is given in Figures 3.1 - 3.4. Each beam was cut to a length of span of 24mm, (i.e. the width of

the block). Each beam was cut to a depth of 2mm. This was set as the cutting distance against the back guard of the oscillating saw, and the breadth was dictated by the width of the hoof wall in that individual animal. Once each individual beam of tissue had been cut, perioplic horn was removed from its dorsal edge and the epidermal laminae were trimmed from its caudal edge using a scalpel. The proximal surface of the beam was identified by placing a tiny blob of marker ink in its proximo-lateral corner. The fresh sample dimensions were measured using electronic calipers, and then the samples were weighed using electronic scales to give their baseline weights. The dimensions and weights of the fresh samples are given in Table 5.2.

5.3.2 Beam sample wrapping and storage

The samples were then immediately wrapped again in three layers of Parafilm. (Parafilm is "M" Laboratory Film. American National Can™). The edges of each layer were overlapped each time to make an airtight seal and the Parafilm was pulled taut during application to mould it to the shape of the sample. Care was taken with any sharp edges to ensure the seal was airtight and that the Parafilm was not broken.

Samples were given identification labels on the Parafilm to record the cut number and they were then placed in plastic bags. The plastic bags were also indelibly labelled to show which pony they had come from. All samples were then returned to the refrigerator at 4°C, to await mechanical testing.

5.3.3 Three point bending of beams

Before being subject to bending tests, all the wrapped beam samples were removed from the fridge, and allowed to equilibrate with the air for 2 hrs. The samples were then unwrapped, one at a time, before bending. The samples were re-weighed and the depth, length and hoof wall width dimensions re-checked. There had been no change in weight nor dimensions of the beams during their ten months of storage from February/March – December/January. The beam bending was done last, once growth and growth rate data, TD and histomorphology data had been assessed from the trial samples during the intervening 10 months.

Table 5.2 Dimensions and weights of beam samples

a) dimensions of fresh beams (mm)

sample: \ dimensions:		Depth	Breadth	Span
Cut 1				
Control	1	2.16	5.77	24.00
	2	1.86	6.33	24.00
	3	2.05	7.58	24.00
	4	2.10	6.48	24.00
Treatment	5	1.92	7.77	24.00
	6	1.80	7.57	24.00
	7	1.94	5.68	24.00
	8	1.97	5.39	24.00
Cut 2				
Control	1	1.83	6.16	24.00
	2	1.80	6.32	24.00
	3	2.15	8.36	24.00
	4	2.37	6.37	24.00
Treatment	5	1.90	7.60	24.00
	6	1.87	7.42	24.00
	7	2.04	5.81	24.00
	8	2.07	5.45	24.00
Cut 3				
Control	1	2.07	6.19	24.00
	2	2.07	6.34	24.00
	3	2.05	8.28	24.00
	4	2.37	6.68	24.00
Treatment	5	1.95	7.57	24.00
	6	1.80	7.46	24.00
	7	2.02	5.95	24.00
	8	2.08	5.59	24.00

b) weights (in grammes) of cut 1,2 and 3 beams in fresh, fully hydrated and dry states

Hydration State	Sample							
	C1	C2	C3	C4	T1	T2	T3	T4
F c1	0.370	0.300	0.413	0.420	0.489	0.380	0.266	0.277
H c1	0.395	0.296	0.467	0.421	0.484	0.416	0.287	0.270
D c1	0.289	0.224	0.359	0.303	0.366	0.306	0.212	0.196
F c2	0.304	0.287	0.531	0.513	0.503	0.401	0.270	0.310
H c2	0.323	0.324	0.541	0.532	0.464	0.429	0.285	0.316
D c2	0.236	0.267	0.418	0.385	0.357	0.351	0.220	0.205
F c3	0.371	0.403	0.530	0.565	0.456	0.381	0.330	0.308
H c3	0.391	0.442	0.552	0.600	0.502	0.423	0.341	0.334
D c3	0.285	0.326	0.435	0.494	0.440	0.311	0.251	0.247

Key: F = Fresh, D = Dry, H = Fully Hydrated
c1 = cut 1, c2 = cut 2, c3 = cut 3
C = Control, T = Treatment

5.3.3.1 Direction of bending of beams

Leach (1980) had investigated the properties of hoof horn by compression testing square blocks in the vertical (proximo-distal) or *y* plane, and in the horizontal (dorso-palmar) *z* plane. In the work for this thesis the bending stiffness of beams of horn were investigated in the *x* plane only.

As explained in section 5.1.4. a span to depth ratio for the beams of greater than 10:1 before mechanical testing is required to remove "end effects" that is, the stiffening effects that having support too close to the middle of the beam would have. This also negates shear effects within the beam (Jackson 1992).

For bending tests in the proximo-distal direction this could be achieved and the beams would lie flat on the bending apparatus supports.

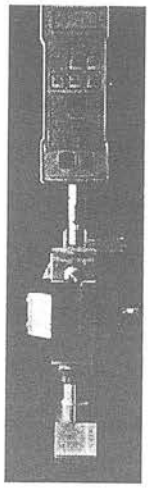
However, were the beams to be placed on the testing rig in the dorso-palmar direction, the samples would not stay on the supports unaided because of their raised centres of gravity and would have fallen over. Free testing could not therefore be carried out on beams placed 'end on' and even if it could have been, say by supporting the beams with glue or 'blue-tac', as this would have increased the artificial effects of support on subsequent stiffness readings, in addition to the erroneous effects of having far too small a span:depth ratio. These effects would have rendered any results from testing in the dorso-palmar direction invalid.

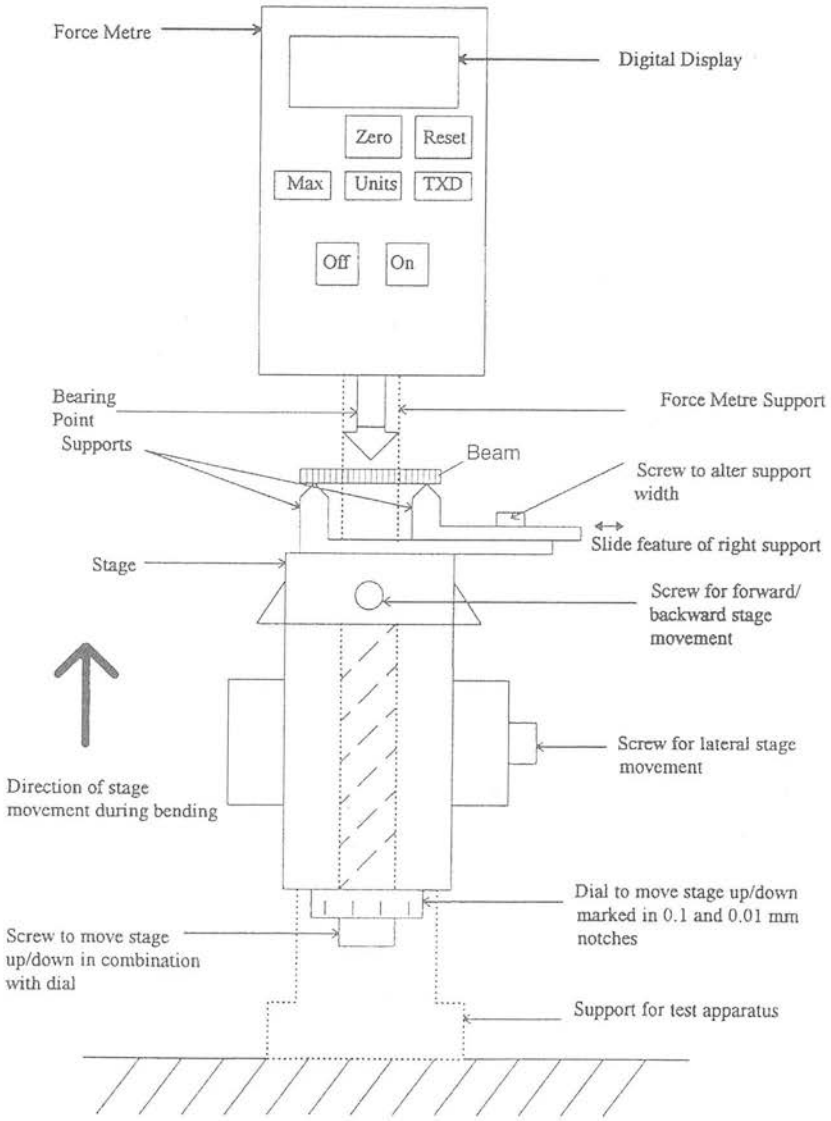
For these reasons testing in the dorso-palmar direction was not carried out.

5.3.3.2 Beam bending protocol

A three point bending technique was used to test hoof stiffness with the beam being supported at both ends and loaded in the middle. The bending apparatus, or 'rig' (Jackson 1992) used is shown in Figure 5.4.

Figure 5.4: Force meter and bending apparatus (not to scale)





Samples were placed flat in the *x* plane and centrally across the supports with the most proximal part of the beam uppermost and the bearing point of the rig centred over the sample.

The bending apparatus was set up with the stage in the centre of its lateral and proximo-distal travel ranges. The span of support was adjusted to 24mm by using the adjusting screw and slide feature of the right hand support (Figure 5.4).

The samples were then subjected to 3 point bend testing in air at room temperature (approximately 20°C), having equilibrated in the room for 2 hrs and immediately after removal of the Parafilm.

The procedure for beam testing was as follows:

The force metre (Mecmesin portable force indicator, Mecmesin PF, Version 1.7, Mecmesin Ltd. Shown in Figure 5.4) was suspended above the 3 point bending apparatus and was set to read grammes force. The force metre was zeroed (by pressing "ZERO") and the sample was pre-loaded to below a maximum of 4g by turning the bottom screw to move the stage up or down. This 'pre-load' was used to minimize the possible effects of movement of the specimen during initial loading and initial specimen curvature when applying a small load (ASTM E855, 1994). This ensures that a 'toe' in the stress strain curve (see Figure 5.1) is avoided as the system 'settles' (Jackson 1992).

A movement dial indicator (Verdict 0.01mm), used to verify the distance through which the sample was bent, was zeroed by holding the bottom stage movement screw still and turning the dial to "0". The force metre was then zeroed and set to read "maximum only" (by pressing "MAX") prior to loading.

The bottom stage movement dial was marked in 0.1 and 0.01mm divisions. A crosshead speed of 2mm/minute (Kitchener 1987) was achieved by manually turning the bottom dial 0.1mm every 3 seconds for 30 seconds until the beam had bent through a total deflection of 1.0mm. The timings were achieved using a stopwatch.

The maximum reading (in grammes force) from the force metre was recorded after each turn of the dial through 0.1mm. If the dial was accidentally advanced too far, or not far enough, it was not readjusted, but advanced after the next 3 seconds to the next 0.1mm division.

Once full deflection bending had been achieved, the dial was then immediately turned back to below zero to remove the load. The final maximum reading given was then cancelled (by pressing "MAX" once), and there then followed a 60 second "rest interval" before the same sample was preloaded again to below 4g, the bending procedure was then repeated for a 2nd and 3rd bend for each beam sample. A simple calibration test for the force meter was carried out between samples by hanging a 1g, 2g and 4g weight from the probe and comparing this to the reading given out on the display. The display reading was always wholly accurate.

5.3.3.3 Manipulation of sample hydration and rebending

The need to control for MC% of the samples was discussed in Section 5.1.5. In the work for this thesis, once the beams had been subject to bending tests in their 'fresh' state, the samples were then taken to a fully hydrated state (100% MC).

In order to find the original moisture content of the hoof horn samples (% MC 'fresh'), this was calculated by comparing the wet weight of the samples with their 'air dried' weights and their 'wet' weights retrospectively. Fresh MC% could then be expressed gravimetrically using either the equilibrated air dried weight (0% MC) or the equilibrated in distilled water weight (100% MC) as a reference point as other authors had done in the past: (Miyaki 1974, Leach 1980, Kitchener 1987, Betram and Gosline 1987).

Hydration - mass changes

Samples were placed in individual pots in distilled water at room temperature (20°C) where they absorbed water to full hydration (100% MC) (Kitchener & Vincent 1987). The individual mass of each sample was recorded daily after excess surface water had been removed by blotting with a paper towel until no further water imprint could be seen on the towel. For all beams, constant mass was reached by 7 days as Kitchener and Vincent (1987) had found.

Wet bending (100% MC)

The 'wet' samples then underwent bending tests, following the protocol already given in section 5.3.3.2. The loss of water to the atmosphere was taken to be negligible during the short period required for the bending procedure, because there was no change in mass of the specimens during this interval.

Drying – mass changes

Samples were then allowed to air dry at room temperature and weighed daily until no further mass loss took place.

Dry bending (0%MC)

The bending procedure was then repeated for the 'dry' samples.

Hydration calculations

The moisture contents of the fresh (FMC%) and hydrated (HMC%) samples were calculated as a percentage of fresh or hydrated weight as follows:

$$FMC\% = \frac{FW - DW}{FW} \times 100\%$$

after Von Bergen (1963)

$$HMC\% = \frac{WW - DW}{WW} \times 100\%$$

after Von Bergen (1963)

Key:

FW = Fresh weight Mass of original sample after unwrapping.

DW = Dry weight Final mass of sample following no further mass loss.

WW = Wet weight Final mass of sample following no further mass gain.

Example of moisture content calculation for fresh cut 1, Control 1:

FW 0.370g

DW 0.289g

WW 0.395g

$$FMC\% = \frac{0.370 - 0.289}{0.370} \times 100\% = 21.89\%$$ (See Table 5.6)

$$HMC\% = \frac{0.395 - 0.289}{0.395} \times 100\% = 26.84\%$$ (See Table 5.6)

Moisture regain

Fresh moisture regain (FMR) and Hydrated moisture regain (HMR) were calculated as follows:

$$FMR = \frac{FW - DW}{DW} \times 100\%$$

After Von Bergen (1963)

$$HMR = \frac{WW - DW}{DW} \times 100\%$$

After Von Bergen (1963)

Example of moisture regain for fresh cut 1, control 1:

FW 0.370g

DW 0.289g

WW 0.395g

$$FMR = \frac{0.370 - 0.289}{0.289} \times 100\% = 28.03\% \text{ (See Table 5.6)}$$

$$HMR = \frac{0.395 - 0.289}{0.289} \times 100\% = 36.68\% \text{ (See Table 5.6)}$$

Statistics

The mean, standard deviation (sd) and Coefficient of Variation (CV) were calculated for the results for bending stiffnesses at three moisture contents ('fresh', 'wet' and 'dry'), as were percentage moisture content (fresh and wet) and percentage moisture regains.

Data were tested for normality using the Kolmogorov-Smirnov normality test on the Minitab Statistical Software Package.

Appropriate parametric or non-parametric tests were then used on the data for 'fresh' bending stiffnesses to see whether there were 'between bends' or 'between cuts' differences, which would then dictate how the rest of the data set could be handled.

5.4 Results

5.4.1 Fresh stiffness (E) data

Figure 5.5 gives an example of a stress-strain plot for beams from control 1 and treatment 1 animals to show that the stress-strain relationships were linear and that the beams showed Hookean behaviour. This was the case for all beams. The calculation of **material** stiffness from these **beam** stiffnesses were calculated as given in section 5.1.4 (Simple beam theory), and an example of this calculation for a control animal is given below:-

Example calculation of E for control 1

For Control 1 Cut 1:

$$I = \frac{bd^3}{12} = \frac{5.77 \times (2.16)^3}{12} = \underline{4.848\text{mm}}$$

$$\therefore 48I = 48\ (4.848)$$
$$= \underline{232.608}\ (\text{mm})$$

$$S^3 = (24)^3 = \underline{13824}\ (\text{mm})$$

F/x (apparent beam stiffness), from slope of stress strain graph (in Figure 5.5)

$$= \underline{1037}\ (\text{g/mm})$$

$$\therefore f/x \times S^3 = 14335488$$

$$\frac{fx/S^3}{48I} = \frac{14335488}{232.608} = 61629.385\ \text{g/mm}^2\ (\equiv E_{\text{material}})$$

$$= 61.629385\text{Kg/mm}^2$$

$$\times 9.8\ (\text{Kg force}) \qquad (1\text{Kg} = 9.806\text{N})$$

$$= \underline{603.96797\ \text{N/mm}^2}$$

to correct $\rightarrow mm^2 \rightarrow m^2 \div$ by 1×10^{-6}

$$= 6 \times 10^8\ \text{N/m}^2$$

$$\therefore E_{\text{material}} = \underline{0.6\ \text{Gpa}}$$

Figure 5.5 Stress-strain plot for sample beams from Control 1 and Treatment 1 animals

```
MTB > mplot c2 c10;c42 c10

      -
 1050+                                                          B
      -                                                       B  A
      -                                                       A
      -                                                    B
      -                                                 2  A
  700+                                                 2
      -                                              2
      -                                           2
      -                                        2
  350+                                                    A = control
      -                                  2                B = treatment
      -                            A                      2  = coincidental plot for control and treatment
      -                            B
      -                  2
    0+
      +---------+---------+---------+---------+---------+------
      0.00      0.20      0.40      0.60      0.80      1.00
      A = C2 vs. beamdfmm       B = C42 vs. beamdfmm
```

grammes force (g) ↑

→ beam deflection (mm)

```
MTB > regress c2 1 c10
```

(ie: regress Control 1 stress v strain)

```
The regression equation is
C2 = - 19.1 + 1037 beamdfmm
```

∴ 1037 g/mm = slope = E_{beam}

Predictor	Coef	Stdev	t-ratio	p
Constant	-19.07	18.88	-1.01	0.342
beamdfmm	1036.85	30.43	34.07	0.000

s = 27.64 R-sq = 99.3% R-sq(adj) = 99.2%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	886920	886920	1160.62	0.000
Error	8	6113	764		
Total	9	893034			

Unusual Observations

Obs.	beamdfmm	C2	Fit	Stdev.Fit	Residual	St.Resid
8	0.80	753.00	810.41	11.59	-57.41	-2.29R

R denotes an obs. with a large st. resid.

```
MTB > regress c42 1 c10
```

(ie: regress Treatment 1 stress v strain)

```
The regression equation is
C42 = - 53.4 + 1138 beamdfmm
```

∴ 1138 g/mm = slope = E_{beam}

Predictor	Coef	Stdev	t-ratio	p
Constant	-53.400	9.750	-5.48	0.000
beamdfmm	1137.64	15.71	72.40	0.000

s = 14.27 R-sq = 99.8% R-sq(adj) = 99.8%

The individual material stiffnesses for control and treatment group samples were calculated from the stress-strain data for the beams obtained for both 0.5 and 1.0 mm beam displacements.

5.4.1.1 Basic descriptive statistics

Analysis of the material stiffness values by Kolmogorov-Smirnov normality testing revealed that the data were normally distributed ($P<0.05$) in both the control and treatment groups at both displacement values. Thus parametric tests could be used in analysis of these data sets.

The control group had a mean material stiffness of 0.5247 GPa (sd +/- 0.1472) with a coefficient of variation (CV) of 0.281 from data generated from the 0.5 mm displacement. Absolute values ranged from 0.2945 to 0.8532 GPa. This compared with a mean material stiffness derived from the 1.0mm displacement data of 0.5166 GPa (sd+/- 0.1373) and a CV of 0.266, with absolute values ranging from 0.2835 to 0.7799.

The corresponding mean material stiffness values in the treatment group were 0.4796 GPa (sd +/- 0.1273) with a CV of 0.265 and 0.4861 GPa (sd +/- 0.1231) and a CV of 0.253 for the 0.5 and 1.0 mm displacement data. Absolute values ranged from 0.2774 to 0.7015 GPa for the 0.5mm displacement and 0.2776 and 0.72293 GPa for 1.0 mm displacement.

5.4.1.2 Differences in stiffness (E) calculated at 0.5mm and 1.0mm displacement.

Since the data were normally distributed a comparison of the material stiffnesses for Treatment and Control groups was carried out by parametric paired t-test. This demonstrated that there was no significant difference ($P>0.05$) between the bending E s for the 0.5 and 1.0 beam displacement data in either the control or treatment group.

5.4.1.3 Differences in stiffness (E) between bends at 0.5mm and 1.0mm displacement

The mean material stiffness for the control group, calculated at both 0.5 and 1.0 mm displacement increased progressively for each respective bend. This is shown (in Table 5.3) below:

Table 5.3: Differences in mean +/-sd stiffness (E) between bends at 0.5 and 1.0mm displacements for the Control group

Control group			
Displacement	Bend 1 (GPa)	Bend 2	Bend 3
0.5 mm	0.4998 +/- .1420	0.5322 +/- .1461	0.5380 +/- .1561
1.0 mm	0.4992 +/- .1351	0.5199 +/- .1423	0.5308 +/- .1447

A similar trend of increasing stiffness with successive bending was recorded for the treatment group, and is shown in Table 5.4 below:

Table 5.4: Differences in mean +/-sd stiffness (E) between bends at 0.5 and 1.0mm displacements for the Treatment group

Treatment group			
Displacement	Bend 1	Bend 2	Bend 3
0.5 mm	0.4403 +/- .1299	0.4919 +/- .1270	0.5072 +/- .1263
1.0 mm	0.4574 +/- .1345	0.4922 +/- .1264	0.5037 +/- .1151

There was no consistent pattern of difference between the absolute stiffness values obtained from the two respective displacements. Visual inspection of the respective force displacement graphs revealed a linear relationship up to 1mm displacement with no obvious yield point.

Differences between bends were investigated using a parametric one-way analysis of variance test (ANOVA) with 'between bend' comparisons evaluated by Tukey testing at a confidence interval of 95%.

ANOVA revealed that there was no significant difference in stiffness between bends ($P>0.05$) as calculated at either 0.5 and 1.0mm displacement in either the control or treatment group.

These results indicate that bending up to 1.0mm displacement gives E values which remain within the linear elastic region for hoof horn material and that no permanent deformation occurred over this range of bending. Since there was no difference between bends on this basis, it was decided that all subsequent comparisons were to be conducted using only bend 1 data calculated at 1.0 mm displacement.

Otherwise to have included the data from bends 1, 2 and 3 in subsequent calculations would have created a pooling fallacy (Scott, G. personal communication, Fielding, H. personal communication and Machlis *et al* 1985)

5.4.1.4 Differences in stiffness (E) between Cuts

The mean stiffnesses for the 3 respective cuts, as derived from bend 1 data to 1.0mm displacement, are shown in Table 5.5 below for both the control and treatment groups.

Table 5.5: Mean +/-sd stiffness (E) by cut for treatment and control group beams:

Control Group		
Cut1	Cut 2	Cut 3
0.4453 +/- .1241	0.4407 +/- .1386	0.6116 +/- 0.0838

Treatment Group		
Cut1	Cut 2	Cut 3
0.4696 +/- .1765	0.4192 +/- .1589	0.4834 +/- .0.830

A comparison between the stiffness values for the 3 cuts was conducted by ANOVA test with 'between cut' comparisons evaluated by Tukey testing at a confidence interval of 95%.

There was no significant difference ($P>0.05$) between cuts in either the control or treatment group.

On this basis all comparisons between treatment and control group stiffness values were based upon bend 1 data (from section 5.4.1.3) and for a single cut (cut 1) at each of the three different hydration levels i.e. fresh, fully hydrated and dry.

5.4.2 Moisture content and regain

The moisture contents and regains of fresh and fully hydrated beams for treatment and control groups are given in Table 5.6.

5.4.2.1 Moisture content values for the control group

The mean moisture content for all beams in the control group was 19.71% (sd +/- 6.13) with a CV of 0.31. Absolute values ranged from 6.97% to 29.24%.

Table 5.6: Moisture contents of fresh and hydrated samples

% Fresh Moisture Regain (FMR)								
(FW-DW)/DW x 100%	Control 1	Control 2	Control 3	Control 4	Treatment 1	Treatment 2	Treatment 3	Treatment 4
F c l	28.03	33.93	15.04	38.61	33.61	24.18	25.47	41.33
F c2	28.81	7.49	27.03	33.25	40.90	14.25	22.73	51.22
F c3	30.18	23.62	21.84	14.37	3.64	22.51	31.47	24.70
% Fresh Moisture Content (FMC %)								
(FW-DW)/FW x 100%								
F c1	21.89	25.33	13.08	27.86	25.15	19.47	20.30	29.24
F c2	22.37	6.97	21.28	24.95	29.03	12.47	18.52	33.87
F c3	23.18	19.11	17.92	12.57	3.51	18.37	23.94	19.81
% Hydrated Moisture Regain (HMR)								
(WW-DW)/DW x 100%								
H c1	36.68	32.14	30.08	38.94	32.24	35.95	35.38	37.76
H c2	36.86	21.35	29.43	38.18	29.97	22.22	29.55	54.15
H c3	37.19	35.58	26.90	21.46	14.09	36.01	35.86	35.22
% Hydrated Moisture Content (HMC %)								
(WW-DW)/HW x 100%								
H c1	26.84	24.32	23.13	28.03	24.38	26.44	26.13	27.41
H c2	26.93	17.59	22.74	27.63	23.06	18.18	22.81	35.13
H c3	27.11	26.24	21.20	17.67	12.35	26.48	26.39	26.05

Key
F: fresh
H: hydrated
W: wet

c1: cut one
c2: cut two
c3: cut three

Control beam moisture content values and regain values were normally distributed P>0.05, as confirmed by the Kolmogorov-Smirnov normality test.

5.4.2.2 Moisture content values for the treatment group

From Table 5.6 the mean 'fresh' moisture content for all beams in the treatment group was 21.14% (sd+/- 8.09). They had a range from 3.51% to 33.87% and a CV of 0.38.

Regain values ranged from 3.64% to 51.22% with a mean of 28.0% (sd +/- 12.79) and a CV of 0.45.

Analysis of Treatment group beam moisture content and regain values by Kolmogorov-Smirnov normality test revealed that the data were normally distributed (P<0.05).

5.4.2.3 Moisture content of differences between fresh cuts

The mean fresh moisture contents for the 3 respective cuts are shown in Table 5.7 below for both the control and treatment groups.

Table 5.7: Mean +/- sd moisture content by cut for treatment and control beams

Control Group Percentage Moisture content		
Cut1	Cut 2	Cut 3
22.04 +/- 6.46	18.90 +/- 8.10	18.20 +/- 4.37

Treatment Group Percentage Moisture Content		
Cut1	Cut 2	Cut 3
23.54 +/- 4.55	23.47 +/- 9.74	16.41 +/- 8.92

Comparison of fresh moisture contents of the 3 cuts by ANOVA test with 'between cut' comparisons evaluated by Tukey testing at a confidence interval of 95%, revealed no significant difference in moisture content (P>0.05) between cuts in either the control or treatment groups, even though the absolute values for moisture content decreased with successive cuts in both treatment and control groups.

The mean moisture regain values for the 3 respective cuts are shown in Table 5.8 below for both the control and treatment groups.

Table 5.8: Mean +/- sd moisture regain by cut for treatment and control beams

Control Group Percentage Moisture Regain		
Cut1	Cut 2	Cut 3
28.90 +/- 10.20	24.15 +/- 11.41	22.50 +/- 6.50

Treatment Group Percentage Moisture Regain		
Cut1	Cut 2	Cut 3
31.15 +/- 7.97	32.29 +/- 16.83	20.58 +/- 11.92

Comparison between the moisture regain values of the 3 cuts was conducted by ANOVA test with 'between cut' comparisons evaluated by Tukey testing at a confidence interval of 95%.

There was no significant difference in percentage moisture regain values between cuts ($P>0.05$) in either the control or treatment groups.

5.4.3 Comparison of fresh stiffness values by group

Group analysis of stiffness values was conducted by paired t-test comparison of the cut 1 bend 1 data. No significant difference was observed between the material stiffness values for the treatment and the control groups. However, the treatment group stiffness value was higher than for the control group (0.516 +/-0.153GPa in the treatment group, compared with 0.457+/-0.117GPa in the control group and in each pair case, the treatment value was higher than the control value.)

The material stiffness values for cut 1 bend 1 beams for control and treatment pairs are given in Table 5.9 below:

Table 5.9: Material E values for fresh cut 1 bend 1 beams for treatment and control pairs

Control material E (Gpa)			
Pair 1	Pair 2	Pair 3	Pair 4
0.616333	0.453926	0.420207	0.338048

Treatment material E (Gpa)			
Pair 1	Pair 2	Pair 3	Pair 4
0.715140	0.536230	0.464169	0.349722

5.4.3.1 Comparison of moisture content and moisture regain by group

The mean moisture content in the cut 1 control group was 22.04% (sd +/- 6.45). This compared with a mean of 23.54% (sd +/- 4.55) within the treatment group.

Mean moisture regain values were 28.90% (sd +/- 10.21) and 31.15% (sd +/- 7.97) in the control and treatment group respectively.

Group analysis of moisture content and regain was conducted by paired t-test comparison of the cut 1 data.

There were no significant differences ($P > 0.05$) between either moisture content or moisture regain between treatment and control groups.

5.4.4 Fully hydrated beam data

5.4.4.1 Differences between cut 1 bends

The mean material stiffness for each respective bend, calculated from both the 0.5 and 1.0 mm displacement data for cut 1 of the treatment and control groups is given in Table 5.10 below:

Table 5.10: Mean (+/-sd) material E values, by cut, for fully hydrated beams in control and treatment groups

Control group	Mean stiffness +/- s.d (GPa)		
Displacement	Bend 1	Bend 2	Bend 3
0.5 mm	0.3450 +/- .051	0.3628 +/- .060	0.3658 +/- .056
1.0 mm	0.3359 +/- .060	0.3472 +/- .072	0.3455 +/- .071

Treatment group	Mean stiffness +/- s.d (GPa)		
Displacement	Bend 1	Bend 2	Bend 3
0.5 mm	0.4058 +/- .065	0.4092 +/- .062	0.4200 +/- .060
1.0 mm	0.3770 +/- .057	0.3808 +/- .060	0.3887 +/- .060

The stiffness values calculated from the 1.0mm displacement data were consistently lower than those based on 0.5mm displacement data in both the treatment and control groups.

Analysis of the stiffness data distribution by Kolmogorov-Smirnov normality test revealed that the data were non-normally distributed ($P<0.05$) in the control group at both displacement values, whereas in the treatment group the data were normally distributed.

5.4.4.2 Stiffness differences calculated at 0.5 and 1.0mm displacement for the control group

The median stiffness derived from 0.5 mm displacement data was 0.34511 GPa, this compared with 0.3206 GPa calculated from the 1.0mm displacement data.

Comparison of the flexural moduli by nonparametric Mann-Whitney test demonstrated that there was no significant difference ($P>0.05$) between bending E's in the control group based upon the 0.5 and 1.0 beam displacement data.

5.4.4.3 Stiffness differences calculated at 0.5mm and 1.0mm displacement for the treatment group

The mean material stiffness from the 0.5mm displacement data was 0.4117 GPa (sd +/- 0.057) with a CV of 0.14. This compared with material stiffness derived from 1.0 mm displacement data of 0.3822 GPa (sd +/- 0.054) and a CV of 0.14.

Comparison of the stiffness values by parametric paired t-test revealed that there was a highly significant difference ($P<0.01$) between the bending moduli based upon the 0.5 and 1.0mm beam displacement data, with the 0.5 mm stiffness values higher than those calculated from the 1.0mm displacement data.

On this basis it was decided to base all subsequent statistical analysis on stiffness values derived from the 0.5mm displacement data because the lower stiffness

values at 1.0mm displacement would be due to a decrease in slope of the stress-strain plot at the higher displacement values, whereas the initial slopes of the graph, to 0.5mm displacement, could be considered comparable.

5.4.4.4 Stiffness differences between control group cut 1 bends at 0.5mm displacement

The mean stiffness values for the control group, calculated at 0.5 mm displacement increased progressively for each respective bend.

Comparison between material stiffness of the 3 respective bends by non parametric Kruskal-Wallis tests, with 'between cut' comparisons evaluated by Mann-Whitney testing at a significance level of 95%, revealed that there were no significant differences between bends ($P>0.5$).

5.4.4.5 Stiffness differences between treatment group cut bends at 0.5mm displacement

The mean stiffness values for the control group, calculated at 0.5 mm displacement increased progressively for each respective bend.

Comparison between the material stiffnesses of the 3 bends was conducted by ANOVA test with 'between cut' comparisons evaluated by Tukey testing at a confidence interval of 95%. There were no significant differences between bends ($P>0.5$).

On this basis, it was decided to base all between group comparisons on cut 1 bend 1 data, as per the convention adopted with the fresh weight samples, with stiffnesses calculated from 0.5 mm displacement data.

5.4.4.6 Moisture content and regain for fully hydrated beams

The mean moisture content of the control group beams was 24.12% (sd +/- 3.72) with a coefficient of variation (CV) of 0.15. Absolute values ranged from 17.59% to 28.03%. This compared with a mean moisture regain of 32.07% (sd +/- 6.27%) and a CV of 0.20, with absolute values ranging from 21.35% to38.94%.

Moisture content and regain values were normally distributed $P>0.05$, as confirmed by the Kolmogorov-Smirnov normality test.

The moisture content in the treatment group ranged from 12.35% to 35.13% with a mean value of 24. 57% (sd +/- 5.48) and a CV of 0.22. Regain values ranged from 22.22% to 54.15% with a mean of 34.03% (sd +/- 12.79) and a CV of 0.38.

Analysis of the Moisture content and regain values by Kolmogorov-Smirnov normality test revealed that the data were normally distributed (P>0.05).

5.4.4.7 Moisture content differences between cuts

The mean moisture contents for the 3 respective cuts are shown in Table 5.11 below for both the control and treatment groups.

Table 5.11: Mean percentage moisture content of fully hydrated beams, by cut, for control and treatment groups

Control Group Percentage Moisture content +/- s.d		
Cut1	Cut 2	Cut 3
25.58 +/- 2.25	23.72 +/- 4.62	23.05 +/- 4.44

Treatment Group Percentage Moisture Content		
Cut1	Cut 2	Cut 3
26.09 +/- 1.26	24.79 +/- 7.25	22.82 +/- 6.98

Comparison between moisture contents for the 3 cuts was conducted by ANOVA test with 'between cut' comparisons evaluated by Tukey testing at a confidence interval of 95%.

There was no significant difference in moisture content (P>0.05) between cuts in either the control or treatment group.

The mean moisture regain values for the 3 respective cuts are shown in Table 5.12 for both the control and treatment groups.

Table 5.12: Mean percentage moisture regain of fully hydrated beams, by cut, for control and treatment groups

Control Group Percentage Moisture Regain +/- s.d		
Cut1	Cut 2	Cut 3
34.46 +/- 4.06	31.45 +/- 7.76	30.28 +/- 7.42

Treatment Group Percentage Moisture Regain		
Cut1	Cut 2	Cut 3
35.33 +/- 2.30	33.97 +/- 13.91	32.80 +/- 5.81

Comparison between the moisture regain values of the 3 cuts was conducted by ANOVA test with 'between cut' comparisons evaluated by Tukey testing at a confidence interval of 95%.

There was no significant difference in percentage moisture regain values between cuts ($P>0.05$) in either the control or treatment groups.

5.4.4.8 Comparison of fully hydrated stiffness values by group

Between group stiffness analysis was conducted by a non-parametric Mann-Whitney test comparison of the cut 1 bend 1 data derived from the 0.5mm displacement values. A significant difference was observed ($P<0.05$) between the fully hydrated beam stiffnesses of the treatment and the control groups. The treatment group beam stiffness was significantly stiffer than that for the control group at 100MC% (median of 0.398 GPa in the treatment group, compared with 0.345 GPa in the control group).

5.4.4.9 Comparison of moisture content and regain by group

The mean moisture content in the cut 1 control group was 25.58% (sd +/- 2.25). This compared with a mean of 26.09% (sd +/- 1.26) within the treatment group. Mean moisture regain values were 34.46% (sd +/- 4.05) and 35.33% (sd +/- 2.30) in the control and treatment groups respectively.

Group analysis of Moisture content and regain was conducted by paired

t-test comparison of the cut 1 data. There was no significant difference (P> 0.05) between either moisture content or moisture regain between treatment and control groups.

5.4.5 Dry beam data

5.4.5.1 Differences in E between cut 1 bends

The mean material stiffnesses found for each respective bend, calculated for both the 0.5 and 1.0 mm displacement data for cut 1 of the treatment and control groups, are given in Table 5.13 below:

Table 5.13: Mean dry material E values, by bend, for 0.5mm and 1.0mm beam displacement, for control and treatment groups

Control group	Material stiffness (GPa)		
Displacement	Bend 1	Bend 2	Bend 3
0.5 mm	1.2759 +/- .163	1.4692 +/- .185	1.4180 +/- .360
1.0 mm	1.3465 +/- .360	1.4620 +/- .234	1.4070 +/- .445

Treatment group	Material stiffness (GPa)		
Displacement	Bend 1	Bend 2	Bend 3
0.5 mm	1.3972 +/- .189	1.3560 +/- .236	1.4530 +/- .281
1.0 mm	1.3970 +/- .245	1.4330 +/- .230	1.4560 +/- .271

There was no consistent pattern of difference between the stiffness values obtained from the two respective displacements.

Analysis of the stiffness data by Kolmogorov-Smirnov normality test revealed that the data were normally distributed (P>0.05) in both the control group and treatment groups.

5.4.5.2 Differences between E calculated at 0.5mm and 1.0mm displacement for the control group

The stiffness values calculated from the 0.5mm displacement data ranged from 0.9167 to 1.7668 GPa with a mean of 1.3878 GPa (sd +/- .2432) and a CV of 0.18. This compared with stiffness values from 1.0 mm displacement data that ranged from 0.7668 to 1.7546 GPa with a mean value of 1.4050 GPa (sd +/- .2776) and a CV of 0.20.

Comparison of the stiffness values by parametric paired t-test demonstrated that there was no significant difference (P>0.05) between the bending E's from the 0.5 and 1.0 beam displacement data.

5.4.5.3 Differences between E calculated at 0.5mm and 1.0mm displacement for the treatment group

The flexural moduli calculated from the 0.5mm displacement data ranged from 1.1248 to 1.8447 GPa with a mean of 1.4023 GPa (sd +/- 0.2195) and a CV of 0.16. This compared with flexural moduli derived from 1.0 mm displacement data that ranged from 1.1366 to 1.8466 GPa with a mean value of 1.4188 GPa (sd +/- .2346) and a CV of 0.17.

Comparison of the flexural moduli by parametric paired t-test revealed that there was no significant difference (P>0.05) between the bending moduli from the 0.5 and 1.0 beam displacement data.

From this, it was decided to base all subsequent statistical analysis on flexural moduli values derived from 1.0mm displacement data.

5.4.5.4 Differences between control group cut 1 bends at 1.0 mm displacement

Comparison between the stiffness values from the 3 bends was conducted by ANOVA test with 'between cut' comparisons evaluated by Tukey testing at a confidence interval of 95%. There was no significant difference between bends (P>0.5).

5.4.5.5 Differences between treatment group cut 1 bends at 0.5 displacement

Comparison between the flexural modulus of the 3 bends was conducted by ANOVA test with 'between cut' comparisons evaluated by Tukey testing at a confidence interval of 95%. There was no significant difference between bends (P>0.5).

On this basis, it was decided to base all 'between group' comparisons on cut 1 bend data, as per the convention adopted with the fresh weight samples, with stiffness values calculated from 1.0mm displacement data.

5.4.5.6 Comparison of dry E by group

The control group had a mean stiffness for bend 1 data of 1.347 GPa (sd +/- 0.144) with a coefficient of variation (CV) of 0.11 based upon 1.0 mm beam

deflection. This compared with a mean stiffness derived from the bend 1 displacement data in the treatment group of 1.397 GPa (sd+/- 0.186) and a CV of 0.13.

Group analysis of flexural modulus was conducted by paired t-test comparison of the cut 1 bend 1 data. There was no significant difference observed ($P>0.05$) between the stiffness values for the treatment and the control group.

5.5 Discussion

As a result of the 3 point bending method used, the stiffness values (Es), given from this work are in the *y* plane for a combination of tubular and intertubular horn. They are values for material, or 'horn stiffness' for everything that makes up the material within the cut beam of horn at the MDC of the hoof capsule at the DLNHG in this experiment.

The values for E given are within the ranges for other biological materials and are within the ranges given by other recent authors (See Table 5.1).

Bertram and Gosline (1987) gave an E value of = 0.410 Gpa for hoof horn. Landeau *et al* (1983) gave hoof horn stiffness results of approximately 87,000 psi, and Leach (1980) gave results of approximately 50,000 psi (30-80,000 psi). Since 1 N/m^2 = 1.45×10^{-4} psi (From Landeau *et al* 1983) then the results in this thesis are in the same order as those of Bertram and Gosline (1987), at 0.4 Gpa, and Landeau *et al* (1983), Leach (1980) and Douglas *et al* (1996). However, they do not agree with the results given by Kitchener and Vincent (1987) whose results for E were in order of magnitude higher than those found in this work. This cannot be explained, especially as the same loading rate of 2mm s^{-1} was used, other than the possible fact that the material stiffness for head horn is genuinely higher than for hoof horn at the same moisture content. The results from earlier authors such as Butler and Hintz (1977), Webb *et al* (1984) and Leach (1980) are of a lower order of magnitude. This could be because a lower cross-head speed was used for testing or the (unspecified) MC% of the material tested was higher than in this work.

Sampling at the DLNHG between pairs was a decision that was taken in order to control for sample location between pairs. In hindsight it may have been better to sample at the equivalent DLNHG for the animal with the slowest hoof horn growth of all the animals in this trial, and to have selected the same point for sampling within the

hoof capsule for all the animals. However, this would have meant making assumptions about the anatomy of the capsule being the same at equivalent points down the hoof capsule between all pony types and this information was not known. In addition, from the results which contributed to Chapter two, the two oldest animals in the trial had very slow hoof growth and sampling at such a high point in the capsule may have introduced other effects such as those associated with immature horn in the larger capsules from the younger, and heavier animals.

Further work is required in the future in order to elucidate the structure of the whole hoof capsule and to investigate relationships between structure and age, bodyweight and size, but since this was not the aim of this thesis, and the trial had been designed on a matched pair basis, the matched pair approach to sampling for data generation was the logical approach to be taken.

Bending studies were justified from the findings of previous work by Hood *et al* (1992), and they have proved to be very useful as a means of quantifying the mechanical parameter stiffness. However, bending studies also have limitations. Since the A.R of the beams needed to be > 10:1 in order to give results that were not confounded by end effects as discussed in section 5.3.3.1, this meant that bending could only take place in the *y* plane. This is not a problem in itself, since the same bending method was applied to all beams and the method was carefully controlled. This means that the results *inter alia* are comparable but it does mean that the interpretation of these results is limited to one plane only within the hoof. The stiffness values given by this method nevertheless give valuable information about the mechanical properties of hoof horn at this site.

Due to the unexpected result that each of the three beams that had been cut gave stiffness values which were not significantly different from one another, this also meant that the data-set generated was limited to only one value per animal in the trial. This had been the case with growth and growth rate values in Chapter 2, TD values in Chapter 3 and morphometric data in Chapter 4. Although a limited data set was expected for all these previous measurements because of their very nature, and the anatomical isotropy of the hoof wall at the MDC, this was not so for the mechanical data because the literature had inferred that the mechanical properties of the hoof

wall, largely modulated by moisture content and possibly by its mineral content, may be different at different points in the hoof capsule. A *non-significant* difference for the stiffness values given between beams that had been cut fresh from the hoof wall was therefore unexpected. What this meant however, was that a 'pooling fallacy' where data are pooled in a larger data set than they should be, had to be avoided. If the data are not significantly different from another, then they simply represent repeated measurements, and thus, if pooled, they would falsely give more power to the statistical analysis (Scott, G personal communication, Fielding, H. personal communication and Machlis *et al* 1985)). This meant that, again, single measurements per animal were the only truly representative data to use in an analysis of a treatment effect, or lack of treatment effect in this work. As a result of these findings, the only way to increase the power of the test between treatment and control animals in such trials would be to increase the number of animals in the trial.

Since moisture content has a direct effect on the stiffness of horn, moisture control prior to bending in this work was essential in order to assess the *material* stiffness in the hoof wall, otherwise conclusions could have been drawn about differences in material stiffness that were *actually* due to differences in moisture content. This mistake has occurred in the past where previous workers have not controlled for moisture content, nor recognised its importance in the results that they have obtained for stiffness using other types of mechanical testing.

The attempts to control moisture content within samples by equilibrating them to 100 MC%, or 'fully hydrated' gave some inconsistencies in results. From Table 5.6 there is an unexplained inconsistency in the fresh moisture content values for Treatment 1, fc3, at 3.51% and for Control 2, fc2, at 6.97% and these low values are reflected in the correspondingly low fresh regain values. Although an increase in E value is expected with decreasing MC%, these samples did not show high E values and thus the incongruity was most likely due to inconsistencies in the air drying method in driving-off held moisture from the structure of the beam. Little work has been done on how moisture is held within the hoof wall, and this is an area for further work.

Despite these inconsistencies, the mean values for MC% were within the ranges found by other workers (eg: Leach 1980, Kitchener and Vincent 1987 and Kasapi and Gosline 1997). Interestingly, the process of saturating the beams in water was seen to shift the distribution of MC% data to non-normal in the treatment group only and to produce beam stiffnesses at 1.0mm deflection that were lower than at 0.5mm deflection.

The tail off in the upper slope of the stress-strain relationship in saturated beams may relate to the incompressibility of water within the structure not allowing a proportionate change in strain in the beam for a corresponding increase in stress, at the higher levels of stress to which the beams were subjected. At the molecular level, it is unknown how water molecules within hoof horn interact with keratin filaments or with the proteins found in filaments or matrix. At the cellular level, it is also unknown how water interacts with hoof horn membranes and with tubular and intertubular horn. It is also unknown in which form the water is held and how it fluxes. These are areas for further work.

In the treatment group only the distribution of fully hydrated MC% data was non-normal. This implied that a change in the moisture-holding properties of biotin-treated beams had taken place. However, it was not reflected in a difference between treatment and control MC% values in either the fresh or fully hydrated states.

Significant differences were seen, however, between the fully hydrated beam stiffness values between treatment and control samples.

Since the difference cannot be accounted for by a change in moisture content then this difference in stiffness may be related to the other treatment related findings in previous chapters such as the change in tubule density in zone 4 (Chapter 3) or a change in marrow size (Chapter 4). Whether there are associations between these parameters and stiffness values will be explored in Chapter 6.

A further possible explanation for the difference between treatment and control stiffness values could be as follows: The large influence of MC% on the stiffness of the beams by saturation may mask more subtle effects of biotin on the molecular properties of keratin filaments or on other parts of the hoof wall such as the amount and proportion of matrix proteins. Once beam moisture content has been

normalised, however, more subtle effects of biotin may be able to be discerned. Since keratin biochemical analysis was not undertaken as part of this study, this can only be a speculative suggestion. Nevertheless the direct correlation between stiffness measurements (in compression) and the sum of high sulphur-content and high glycinetyrosine-content proteins in keratinous structures has been shown by Bendit and Gillespie (1978), and Fritsche (1990) and Fritsche *et al* (1991) have shown that pharmacological doses of biotin increase cytokeratin production in *in vitro* keratinocytes. Since, as Webb *et al* (1984) discuss, the mechanical properties of other tissues, such as collagen in connective tissues are dependent on fibril diameters, then the study of hoof horn keratin fibril anatomy and biochemistry, their relationship with mechanical properties and the influence of biotin supplementation on them, are all areas for further study.

There is another approach to understanding the subsequent effects of a given change in a material property of hoof horn, such as a change in stiffness, and that is to use modelling techniques such as Finite Element Analysis (FEA).

The dimensions and movements of the gross hoof capsule were not considered in the work for this thesis, but in a follow-up study to this work an FEA of static loading in the donkey hoof wall was conducted by Newlyn *et al* (1998). (See Appendix VI).

The aim of the study by Newlyn et al (1998) was to design an FEA model for a donkey hoof capsule and to use it to assess whether the theoretical movement of the capsule (as predicted by the model) would comply, or not comply, with that which had been both suggested and objectively recorded by others such as Lungwitz (1891), Leach (1980) and Thomason *et al* (1992).

Newlyn *et al* (1998) found that their theoretical model of gross hoof capsule movement with static loading complied, in general terms, with the practical findings of previous authors. However, in order to create the FEA model a stiffness had to be assumed for donkey hoof horn, as none was available from the literature. This lack of information to feed into a model illustrates that there is much basic work to do in order to refine this approach to investigation. A priority for future research effort is therefore the development of an FEA model for the pony and horse hoof capsules. The results from research such as the work given in this thesis, which has revealed a treatment effect on hoof horn stiffness at full hydration at the MDC, could then be fed into an FEA model to predict the practical effects of such a change on, for example, the stress-strain relationships within the macroscopic hoof capsule.

Newlyn *et al* (1998) also suggested that their model could be developed further to predict mechanical changes, with static loading, at the microscopiclevel of the hoof horn structural hierarchy given in Figure 1.6. Although an average value for hoof wall stiffness had to be assumed for the gross model given in Newlyn *et al* (1998), in a further development of this work, Newlyn et al (1999) gave a microscopic FEA model of the outer aspect of the donkey hoof wall *stratum medium*. (See Appendix VII). The relative stiffness of tubular and inter-tubular material will have important effects on stress and strain distribution at the micro-architectural level in the hoof wall. Newlyn *et al* (1999) found that a hoof tubule to inter-tubular modulus ratio of between 3.5-4.0 gave theoretically modelled results for the material properties of the hoof wall which were in broad agreement with those reported by other workers such as Douglas *et al* (1996). In addition, the position of maximum strain during static loading was found to be located at the boundary of the marrow cavity normal to the direction of loading.

Parallel modelling developments for the horse and pony hoof walls would allow data from work of the type given in this thesis to be used to predict the micro stress-strain concentrations which are developed as a consequence of normal micro-structural organization. Furthermore, models could be used to predict the effects of changes reported in this thesis such as the biotin-induced change in TD reported in Chapter 3 and the biotin-induced change in marrow size reported in Chapter 4. The implications for such predictive abilities are far – reaching. With sufficient development, modelling could be used in the future to tailor the effects of specific nutritional and farriery interventions in hoof horn management.

5.6 Conclusions

The horse hoof has been shown to behave as a linear elastic material during mechanical testing, displaying a Hookean response with stiffness values in the ranges given by Zoerb and Leach (1978), Leach (1980), Landeau *et al* (1983) and Douglas *et al* (1996). In this way, 3-point bending is a useful means of quantifying the mechanical property of stiffness although it has limitations.

Biotin supplementation was found to increase the stiffness of hoof horn in the fully hydrated state

FEA modelling will be a useful means of predicting the effects of a change in hoof horn material properties at the micro and macro levels of structural organization of the hoof capsule.

ADDENDUM TO CHAPTER 5

Introduction

The purpose of this addendum is to give further information about the follow up study into computer modelling of the stress and strain distribution at the micro-architectural level in the hoof wall conducted by Newlyn *et al.* (1999), referred to in Section 5.5, page 233, of this thesis.

The ability to conduct finite element analysis (FEA), and to be able to computer model the macro and micro-architecture of the hoof wall capsule, had been a long standing wish of the author of this thesis (JDR). Dr Hugh Newlyn (HN), a Senior Lecturer in the Department of Engineering at De Montfort University (DMU) Leicester, and a post-graduate student within the hoof group at DMU, Simon Collins (SNC), had been using FEA as applied to the hoof wall. JDR was not involved in the FEA construction or analysis but was heavily involved in discussion of the results and in writing the subsequent published paper.

Materials and Methods

A finite element model (See Fig 1) of the structural and geometric arrangement of the hoof wall was developed. In developing this model, the microstructure of the hoof wall was considered as a 2 component (phase) hollow fibre reinforced composite. Each phase was treated as an isotropic linearly elastic component with perfect bonding assumed at the interface between the horn tubules and the intertubular horn. The geometric structure was simplified by assuming a hexagonal array of repeating horn tubule units each possessing an elliptical cross section in line with the mean major to minor axis ratio. The absolute dimensions of the horn tubule, cortex and medullary cavity were similarly based on the mean recorded values. The inter-fibre spacing combined with the absolute dimensions, was used to generate a fibre density (tubule density) consistent with that known for donkey hoof.

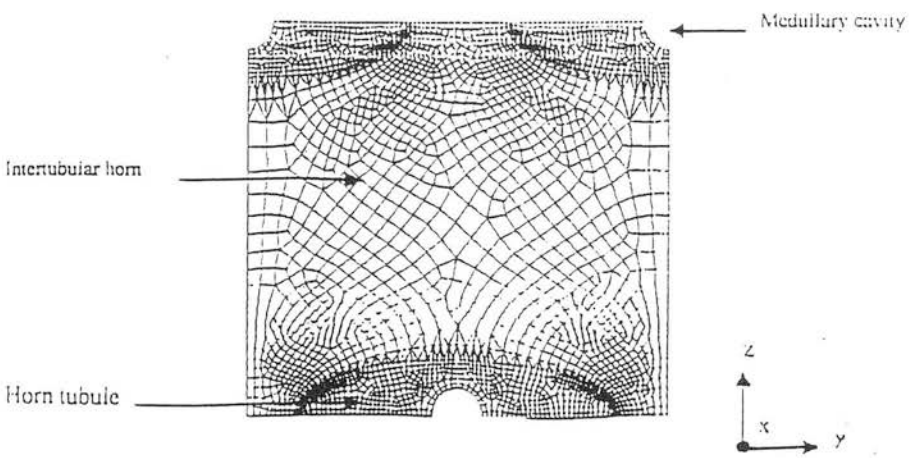


Figure1 - A simplified 2-phase finite element microstructural model of a transverse section of the 'outer' hoof wall region to show the structural and geometric organisation of the horn tubules and the intertubular horn.

Results

1. The relative moduli (stiffnesses) of tubular and inter-tubular horn are not know, because no one has been able to dissect-out and test individual tubules. The FEA model however, indicated that for zone 1 of the donkey hoof wall only, a hoof tubule to inter-tubular modulus ratio of between 3.5 - 4.0 gave results for the material properties of the hoof wall which were in broad agreement with those reported by other workers.

2. The position of maximum strain during static loading was found to be located at the boundary of the marrow cavity perpendicular to the direction of loading.

Conclusions

The major conclusions from this piece of work were that FEA modelling is a useful means of predicting the effect of change of hoof horn material properties at the micro-level of structural organisation. It allows theoretical prediction of concepts that are difficult, at this stage, to explore practically (such as the modulus of tubular hoof horn), and allows a means by which the effects that are brought about by nutritional change, such as changes in tubule density or marrow size, may be modelled.

CHAPTER SIX

Correlations between measured parameters and conclusions of the thesis

6.1 Introduction

In order to investigate whether there were any correlations between the hoof horn parameters that had been measured in the work for this thesis, and in order to see whether these correlated with other pony variables such as, for example, age and bodyweight (weight), a Pearson Correlation Coefficient Matrix was created. This is given in Appendix IV. The correlation coefficient (corr), which is the first figure quoted in each cell of the matrix, gives the degree of correlation which exists between the parameters. The probability of the relationship being due to chance is given by the P value, which is the lower figure in each cell.

The correlation matrix was composed using the data sets for both treatment and control animals together and was therefore representative of the 'stacked' data with no group distinctions. For each case in which a significant correlation was found, the relationship between the two parameters was plotted graphically to ensure that there were no outliers. This condition was satisfied for all significant correlations. These were taken to be those which had P values of <0.05. Only the significant correlations which were found in this way will be discussed in this chapter.

6.2 Correlations between measured parameters and inter-zonal correlations

From the correlation coefficient matrix in Appendix IV, growth and growth rates of the hoof wall at the MDC, in certain periods of the trial, were significantly correlated with other anatomical parameters: Marrow size in Zone 1 of the *stratum medium* had a significant negative correlation with growth and growth rate of hoof horn in period 5 of the trial only. This would suggest that faster horn growth leads to a smaller marrow size and has implications for the nutritional demands made along the length of the papilla during fast growth. However, the horn sampled by the method given in Chapter 3 did not relate to horn grown in period 5 of the trial. Thus, it can be assumed that the treatment effect demonstrated in Chapter 2, which brought about a reduced marrow size in Zone 1 of the *stratum medium* , was caused by some other biotin effect other than that which may have been indirectly caused by a faster growth rate. Nevertheless, future work should explore the effect of growth rate on the anatomical parameters of the hoof wall, and should, perhaps, control for it. There were other similar significant correlations, but for different areas of the stratum medium, for example, growth and growth rates of horn in periods four and five of the trial were significantly correlated to marrow size in Zone 3 only of the *stratum medium* (corr=0.804,P=0.016).

There were numerous examples from Appendix IV of significant correlations between anatomical parameters across zones. For example, median cortex area in Zone 3 and marrow area in Zone 1 had a correlation of 0.850 and a P value of 0.007. Marrow absolute area in Z4 was significantly correlated with absolute marrow area in Zone 1(corr: 0.739, P=0.036) and with absolute cortex area in Zone 1 (corr: 0.717, P=0.045). The area fraction of marrow in Zone 1 was significantly correlated with absolute cortical area in Zone 3 (corr: 0.843, P=0.009).

Such correlations serve to illustrate that there are relationships between anatomical features of the hoof wall between zones. These relationships may exist as a means of diverse biological control, by allowing the possibility of achieving any given biological requirement, such as a certain hoof wall stiffness for example, through a number of subtly different mechanisms. The correlates with hoof wall stiffness will be discussed in Section 6.4, but future work should explore these inter-relationships between anatomical parameters in order to further an understanding of how they may be controlled.

6.3 Pony age and weight related correlations

In Chapter 2 an association between pony age and growth of horn was demonstrated, with the oldest pair of ponies in the trial having a significantly lower hoof growth when compared with the rest ($p<0.05$ by ANOVA).

In Chapter 4 an age-dependent biotin effect was demonstrated, with the younger animals in the trial contributing the greatest effect to a biotin induced decrease in tubule marrow size ($p<0.01$ by ANOVA).

From the correlation coefficient matrix in Appendix IV, both pony age and bodyweight were significantly correlated with absolute tubule cortex size in Zone 2 of the *stratum medium* only. The age correlation with absolute cortex size was a negative one: -0.863, P=0.006 and the weight correlation with absolute cortex size in Zone 2 was a positive one: 0.878, P=0.004.

There was also a significant correlation between bodyweight only and median tubule cortex size in Zone 1 and with absolute tubule size in Zone 3:

Z1 corr 0.710, P=0.049

Z3 corr 0.743, P=0.035.

Interestingly, in Zone 4 a correlation between cortex size and weight and cortex size and age was not seen to exist. However, *marrow* size was significantly correlated with bodyweight in this zone: corr 0.844, P=0.008. Marrow size was also seen to be significantly, but less strongly, correlated with age in Zone 4 only: corr -0.764, P=0.027.

This implies that pony bodyweight, more so than age, may dictate tubule cortex size in Zones 1, 2 and 3 of the *stratum medium* but that in Zone 4 different mechanisms for determining hoof horn function may exist. The implication that the mechanisms of hoof horn function are different by region within hoof wall depth, supports the idea of zonation within the hoof wall and the laminar way in which it may be built up.

From Appendix IV there was also a significant correlation between bodyweight and mean tubule density in Zones 2 and 3 of the stratum medium only (corr: -0.839,P=0.009 and -0.750, P=0.032 repectively). The correlation was highest in Zone 2 but the finer detail of the relationship may be blurred by the artificial division between zones. In the same way as tubule cortex size in Zone 2 has been implicated in the bending mechanism for dealing with static stress (produced as a function of bodyweight), so the spatial arrangement of tubules within the hoof wall (represented by mean TD in Zones 2 and 3) may be important for stress or strain transfer across the *stratum medium*. The fact that this relationship only holds true for two out of the four zones of the hoof wall, and the fact that there was no demonstrable relationship between TD and stiffness, implies that the step-wise reduction in TD from the outer wall to the inner wall may not produce the simplistic mechanism for stress transfer which was suggested in Chapter 3, but that the hoof wall's method of dealing with stress transfer may be more complex than this.

This thesis concentrated on bending studies of whole hoof wall width beams. It is suggested that future work should explore the properties of beams that have been cut into zonal widths, to give information about the relationships between stiffness and anatomical properties that may hold under these conditions. Challenges to such work will be to cut the beams into zones without significant loss of material aswell as maintaining the necessary aspect ratio for bending studies, whilst using a bending rig of sufficient sensitivity to gain results.

From the significant cross-correlations between anatomical features given in Section 6.2 above, aswell as the demonstrated relationships between anatomical features and bodyweight, it is not unreasonable to assume that there may be different biological feed-back mechanisms operating within the hoof wall which may allow biological control of material properties through organic responsive change. It could be speculated, for example, that the dermal papillae may be stress (bodyweight) and/or strain-responsive organs that are able to manufacture tubule components of a required size and density to cope with differing environmental or physiological conditions, and that they are also able to interpret information about other zones of the hoof wall and produce reponsive change accordingly. Whether this is true, and how such responses may be achieved at the cellular level, requires further work and experimentation. How this work

is achieved represents an experimental challenge as it entails working at the dermo-epidermal junction rather than with hard keratinized tissue alone. Methods need to be developed for measuring dermal papillae sizes and for imaging and measuring their cellularity. Stereological measurements taken from morbid specimens from treatment and control animals which have been subject to different stimuli could be compared. Alternatively, *in vitro* methods could be developed such that dermo-epidermal tissue blocks could be sustained under laboratory conditions and physiological and biochemical parameters could be measured while, for example, they undergo representative mechanical, physiological or nutritional stimulation.

The correlation coefficient matrix gives a correlation of -0.921 between age and the fresh stiffness values found in Chapter 5, with a probability of 0.001. However, there is also a positive correlation of 0.729 between bodyweight and fresh hoof horn stiffness with a probability of 0.040. From this, it could be argued that a mechanism by which the animal may be "held up on its feet" may have been revealed:

If heavier animals have higher fresh hoof horn stiffness values then this could allow a comparable relative strain in the hoof wall for a higher static stress caused by a higher bodyweight. However, the highly significant negative correlation between age and hoof horn stiffness means that the older animals in this trial also had less stiff horn. Unfortunately, whether these two relationships between hoof horn stiffness and age, and hoof horn stiffness and weight, are independent or not cannot be unravelled from the results of this trial. This is because the variables of age and bodyweight were confounded in the trial design: The younger animals were also the heaviest and the eldest were the lightest (see Table 2.1).This was unavoidable in view of the limited supply of ponies for purchase which could be match-paired prior to the start of the trial. Future work should therefore aim to seperate these two possible inter-relationships by analysing data gathered from a trial design which allows a spread of bodyweights for the same age and *vice versa.*

Such work would then allow the strength of these two relationships to be tested and a predictive model could then be used to assess whether or not a given individual had the required hoof horn stiffness for its bodyweight or age. Such predictive ability would hold exciting possibilities for problem diagnosis or for corrective therapy. However, it must be remembered that this work was conducted, and initial future work should also be conducted, with normal individuals in order to re-test whether such relationships exist. Assessment of abnormal individuals could then take place and the results compared to those that would then be available from a sound normal data base.

As explained in Chapter 5, care must be taken during the interpretation of hoof horn stiffness results, to ensure that allowance has been made for moisture content, and

that it should be controlled under experimental conditions. There were no significant correlations between age and weight and moisture content. However, a correlation still existed between age and hoof horn stiffness when controlled for moisture content using hydrated stiffness values (Hb). From Appendix IV the correlation between fully hydrated stiffness and age was 0.755 with a P value of 0.030. However there was no correlation between bodyweight and hydrated stiffness value. This implies that there may be an age-related effect on the interaction between moisture content and hoof horn stiffness ie: that the effect of moisture content on reducing hoof horn stiffness diminishes, or is more variable, with age. Control mechanisms for moisture content within the hoof and how these may vary with age should be the subjects of further study.

There were no correlations between age and weight and dry bending stiffness values. In fact there were no relationships found for dry stiffnesses and any other hoof horn parameter. It is unlikely that the material in dry bending was completely devoid of moisture as it was simply allowed to air dry until a stable weight had been assumed. The way in which water was bound within the beam was not investigated in this thesis and is an area for further study. Challenges to overcome in this field are the identification of water molecules in different bound states and their susceptibility to imaging within the structure of hoof horn. Such study would further an understanding of how moisture interacts with the structure. This is clearly important as the Pearson correlation coefficient matrix has revealed highly probable relationships that exist in the results from this work, between wet and fresh stiffnesses and other variables which will now be discussed:

6.4 Hoof horn stiffness and its other correlates

The correlations matrix gave a relationship between bending stiffness of pony hoof horn and the median and absolute sizes of its tubule cortex component in Zone 2 of the *stratum medium* only. The relationship existed for 'fresh' and 'wet' bends, only, as follows:

Fb1 : absolute and median cortical area in Z2: corr=0.80, P=0.016

Hb1 : absolute and median cortical area in Z2: corr =0.80, P=0.020

In addition, the area fraction of tubule cortex in Zone 2 was also significantly correlated to fully hydrated bending stiffness with a correlation of 0.792 and a probability of 0.019.

The fact that tubule area and area fraction correlates with bending stiffness shows that for this zone of the *stratum medium* the tubule cortex has a function in determining stiffness. It also gives an indication of the composite function of hoof wall components. It is perhaps surprising that a Zone 2 parameter was found to correlate with

stiffness, rather than a Zone 1 parameter: An increase in tubule size in a zone which is relatively close to the neutral axis will have a smaller effect on hoof wall bending stiffness, because of the reduced effect of the moment of inertia, I, than would a correlation with, for example, Zone 1 tubule area. Nevertheless the fact, from Section 6.3 above, that tubule cortex size in Zone 2 is correlated to both bending stiffness and age indicates that this an important parameter, and an important zone for dictating pony hoof horn function.

There were no significant correlations between 'dry' bending stiffnesses and any other hoof horn parameter for any zones of the *stratum medium*.

Fresh bending stiffness was negatively correlated to mean TD in Zone 3 of the *stratum medium* only:

Fb1 : Z3MEAN corr -0.760, P=0.029.

This was an unexpected result. Intuitively, stiffness was expected to be positively correlated with TD. However, from Appendix IV, TD was found to have a significant negative correlation with the absolute and mean sizes of tubules within each of the zones of the *stratum medium* (with the notable exception of Zone 4). This means that, for Zones 1, 2 and 3, as TD increases then absolute tubular size decreases and since it appears that, in Zone 2 at least, tubule size is dictating bending stiffness, then this explains why TD is negatively correlated to stiffness.

Hydrated bending stiffness was also significantly correlated to the area fraction of tubule cortex in Zone 2 of the *stratum medium* only:

Hb1 : ZAFCOZ2 corr 0.792, P=0.019

Again, Zone 2 appears to be functionally important in determining bending stiffness of the *stratum medium* and stiffness is seen to be related to a composite parameter within this zone only.

Hydrated bending stiffness was also significantly correlated to absolute marrow area in Zone 4 of the *stratum medium* only:

Hb1: absolute marrow area in Zone 4 (MAZ4): corr 0.774, p=0.024

This shows that the marrow appears to function differently in Zone 4 compared to other zones for which no relationship between marrow measurement and stiffness has been demonstrated. In addition, unlike the situation above where a tubular component of the *stratum medium*, either cortical size or cortical area fraction, is positively correlated with stiffness, it is an unexpected result that a void area should be positively correlated with stiffness.

Zone 4 appears to be exceptional in that it does not have the inverse relationship between TD and tubular size that the other zones show and there appears to be a relationship between marrow size and stiffness in this zone only. From Chapter 4, Zone

4 was seen to have tubules of much larger cortical area than the other zones and Leach (1980) suggested that marrows concentrated stress in the hoof wall structure and also suggested that the thickness of the tubule cortex serves to minimize the transfer of stress to the marrow space. This may be a critical consideration as it has been suggested that the inner hoof wall is subjected to tensile forces during loading (Bertram and Gosline 1986). Wainwright *et al* (1976) stated that , under tensile loading, void space stress concentration more readily initiates crack initiation. Thus the reduction in marrow area fraction across the hoof wall, and the increase in cortex size across the hoof wall, both demonstrated in Chapter 4, may minimize the risk of crack initiation at depth.

The size and shape of the marrow void has other important implications. Stress concentration is also dependent upon the shape of the void space (Gordon 1976). The stress raising effect of an ellipsoid is significantly greater along its major axis than a circular void of similar area fraction (Vogel 1989). In Zone 1 the tubules are oval in shape and are therefore more prone to failure by crack initiation than those in Zone 4. However, from Chapter 4, the treatment effect on marrow size in Zone 1 will theoretically decrease their stress raising propensity, and furthermore, the accompanying increase in cortical size will minimize the transfer of stress to those marrows. Also, the skew in the distribution of marrow size seen in Chapter 4 could indicate that there is a maximum functional size required, for example, to prevent critical stress concentration in the outer zone of the hoof wall as a result of bending forces.

Considering the hoof wall depth as a whole, if the wall is acting as a biological composite, then the spatial distribution of the cortical component of the tubules can be thought of as a fibre component at the structural level . (This is in contrast to keratin fibres acting as a fibre component at the intracellular level). The treatment effect on marrow size, demonstrated in Chapter 4, occured in Zone 1 tubules which are the furthest away from the neutral axis within the whole hoof wall depth. The accompanying increase in cortical (fibre) material, at this position within the wall, maximizes the effect of second moment of area and would mean that the treatment effect upon stiffness of the wall would be theoretically maximal.

In this way the change in marrow size with treatment, and its possible effects on the mechanics of the hoof wall, may explain the increase in hoof wall hardness with biotin supplementation that has been detected by other workers in the past (Webb, Penny and Johnston 1984, Reilly and Brooks 1990, Buffa *et al* 1996). In addition, this finding may help explain the phenomenon of an increase in hardness of the hoof walls of biotin-supplemented animals which cannot be explained by the extent of new horn growth (Simmins and Brooks 1980, Reilly and Brooks 1990): A decrease in the internal dimensions of the marrow could conceivably increase the stiffness of the tubule along its

entire length. Leach (1990) refers to conical shapes as being particularly resistant to bending. Assuming the response to biotin is progressive then, over time, the distally moving marrow and cortex of a biotin responding Zone 1 tubule will have conical shapes along their lengths. In this way the longitudional bending stiffness of the tubule will be increased and this will be reflected distally. Such changes, which lead to a decreased propensity for crack initiation may also explain the findings of Brooks, Smith and Irwin (1977) and Brooks and Simmins (1980) that biotin fed pigs had fewer crack-type lesions on their hoof walls.

Interestingly, Brooks and Simmins (1980) also found that biotin fed gilts, from approximately seven months of age and followed through their subsequent three parities, which had cracks on their hoof walls, were more responsive to therapy than older sows. In addition, the TD changes reported by Kempson, Currie and Johnston (1989) were also in young (weaner) pigs. Johnston and Penny (1989) also found that both age and bodyweight had an effects on the growth and wear properties of pig hoof horn. The effects of age and bodyweight on hoof horn parameters that have been highlighted by this study and by others are clearly areas for further research.

6.5 Summary of findings and final conclusions of thesis

The literature review in Chapter 1 of this thesis had shown that there was little quantitative understanding of the anatomy of the equid SM nor was there any great understanding of the functional role of its components.

This thesis has contributed to knowledge by giving methods by which quantitative aspects of the physiology, anatomy and mechanics of the hoof wall at the MDC of normal ponies can be assessed. These methods have been used to quantify the effects of supplementary dietary biotin, at a single dose rate, on the hoof horn of ponies in a match-paired feeding experiment , to test the null hypothesis that :

"Dietary supplementation with biotin at a dose level of 0.12mg/Kg BW daily has no effect on the physiology, anatomy or mechanics of pony hoof horn".

The null hypothesis has been rejected through the work in this thesis.
Firstly, biotin supplementation was found to cause a significantly higher growth and growth rate of horn at the MDC in treatment compared to control ponies. Although the results for biotin effects on growth of horn in the literature had been equivocal, the results in this thesis agreed with the findings of Buffa *et al* (1992). Further work, at the biochemical and cellular level, is suggested in order to understand the mechanisms for this result. It was also important to know the extent of new horn growth, between individuals of a pair, in order to sample like horn for subsequent testing.

The two older animals in the trial were found to have significantly lower hoof growth when compared with the rest, and age-related responses and correlations have been a feature of the findings in this work.

There was no significant difference in growth or growth rate of horn between feet in this trial and so the left fore foot of each animal was used, following the convention of previous workers (Webb, Penny and Johnston 1984, Buffa *et al* 1992 and Barr *et al* 1995), to give material for anatomical and mechanical studies.

Biotin supplementation was found to increase the material stiffness of beams of horn in three-point bending, but this difference was only expressed in beams in the fully hydrated state. The reasons for this finding are not clear at present, but Johnston (1991) made a similar observation when biotin-supplemented horses were seen to exhibit harder hoof horn during a period of wet weather only, when compared to non-supplemented controls. Further work at the molecular level could determine whether biotin has had an effect on keratin protein structure. In addition, investigation of the relationships between hoof horn biochemistry and anatomy, including keratin type and quantity, and moisture content should be an area for future study.

By quantifying the number of hoof horn tubules per square millimetre of SM (TD), the hoof wall at the MDC was seen to consist of four distinct zones. This was new, quantitative anatomical information which objectively defined previous descriptive reports of the spatial distribution of tubules by HWD within the *stratum medium*.for-which no data had been given. A simple model of the way in which stress may be transferred across the hoof wall which was assumed from this quantified pattern of TD was subsequently not borne out by correlations between bodyweight, hoof wall stiffness and other hoof wall component features.

Biotin supplementation was found to significantly increase TD in Zone 4 of the SM. An increase in whole hoof tubule density had been reported before by Kempson *et al* (1989), in pigs, and by Dittrich *et al* (1994) with horses. In this work a TD change was effected in one part of the SM only. Attempts to relate this to the biotin-induced increase in stiffness are frustrated by the fact that TD was negatively correlated with stiffness according to the Pearson correlation coefficient matrix.

Biotin supplementation was found to cause a decrease in mean marrow size of Zone 1 tubules in treatment compared to control animals in the two younger pairs of ponies. This quantitative finding supports the field observations made from one horse by Geyer *et al* (1988), and is a theoretically plausible method of increasing hoof horn bending stiffness.

However, a definitive conclusion as to whether the decrease in Zone 1 marrow size *accounts* for the stiffness changes recorded in this work cannot be drawn from the

results presented because marrow size in Zone 1 and bending stiffness were not significantly correlated. However, the results in this thesis use whole beam stiffnesses as their terms of reference and so an effect at the zonal level could have been masked as a result of dealing with data generated from mechanical testing using whole beams..

The results from this work have revealed the importance of zonal features of the SM of pony hoof. Future work should be directed at the zonal level of scale in order to test for more direct relationships between structure and function. It is likely that stiffness differences will be found by depth within the hoof wall, but these can then be related to zonal composite components and the whole can then be built into, and tested against, a model of initially part wall, and then whole wall, and eventually whole hoof function. Finite element analysis is a plausible method by which this could be developed. Such an approach will require a multi-disciplinary effort and work at many different levels of the structural hierarchy of the hoof wall in order to successfully unravel other aspects of its structure and function. Nevertheless the evidence given in this thesis which rejects the null hypothesis and gives support to the existence of a 'nutrient hoof-horn axis' also reveals potentially interesting determinants of hoof wall function: marrows in Zone 1, cortices in Zone 2, tubule density in Zones 3 and 4 and marrows again in Zone 4.

The relationship between bodyweight and/or age and hoof wall anatomy, which was confounded in the work for this thesis because the younger animals in the trial were also the heaviest, also needs further clarification.

The effects of other nutrients on hoof wall properties also needs to be tested in double-blind, placebo-controlled, cross-over trials. In order to test the effects of another nutrient, a trial was conducted to assess the effects of an orally-supplemented evening primrose oil mixture (EPOM) on hoof horn structure and function in Army horses by Reilly *et al* (1998c).The published paper is given in Appendix VIII and includes data about lipid content in the normal hoof wall as this, too, may be a determinant of function. Twelve horses were paired as closely as possible according to sex, age, weight, height and colour and then one from each pair was randomly allocated to treatment or control groups. The treatment group received 30mls of oral EPOM per day for approximately five and a half months, otherwise the nutrition and management regimes were the same for all horses. No significant differences ($p > 0.05$) were seen between treatment and control groups for hoof horn growth or growth rate. However, there was a significant difference ($p < 0.05$) in hoof horn growth, within the treatment group only, between weeks four and eight after the start of supplementation. The timing of the response in hoof growth, in the treatment group only, with EPOM supplementation reported by Reilly et al (1998c) was in agreement with known results for skin and nail in human patients supplemented with evening primrose oil. However, it contrasted

with the type, and timing, of another quite different hoof growth effect with biotin supplementation which had been reported by Buffa *et al* (1992) and Reilly *et al* (1998a). This revealed that the hoof capsule is capable of different growth responses when the equid is supplemented with different nutrients.

No significant differences ($p > 0.05$) were seen between treatment and control groups for any of the lipid fractions measured for the *stratum medium* from clippings of the hoof wall by Reilly *et al* (1998c). However, there were substantial differences in perioplic horn lipid analyses with significant increases ($p < 0.05$) in cholesterol esters and partial glycerides and a highly significant reduction ($p < 0.001$) in free cholesterol in the treatment group compared to the controls following EPOM supplementation. This was the first report of supplementation giving rise to a change in the lipid composition of the hoof and it provided direct evidence for a nutrient hoof horn axis (Reilly *et al* 1998c).

Reilly *et al* (1998c) discussed the possible effects of these changes on the biochemistry of the hoof wall keratinocyte cell membrane, in terms of them potentially affecting cell membrane fluidity and the receptiveness of the keratinocyte to biochemical regulators. Future work is justified which aims to investigate the mechanisms for cellular and molecular control and regulation of nutritional responses at the level of the keratinocyte.

This thesis gives evidence for the existence of a 'nutrient hoof horn axis' in the horse and the pony. However, nutritional trials need to be repeated in other ponies and horses, and in other hooved animals, to assess whether the responses are reproducible under differing conditions. The notion of objective testing for nutritional effects on epidermal (or dermo-epidermal) systems could also be widened in future to include monitoring for effects in the skin and coat of all species of veterinary interest.

Our understanding of hoof wall responses in health, disease, and in response to nutrition, will improve through sustained work at all levels of the hoof wall structural hierarchy, from the molecular to the mechanical. The potential exists to use this knowledge to provide diagnostic and treatment interventions in hoof horn management from the level of the fibril to farriery and, in this way, improve the welfare and care of all hooved animals.

CHAPTER SEVEN

Enumerated conclusions from the thesis

The work for this thesis has made original contributions to scientific endeavour and to knowledge in the following ways:

1. The field trial was of a unique design in that biotin at a set dose rate within a pelleted feed had never before been fed experimentally to such a closely controlled group of ponies.

2. The confusion between the use of the terms 'growth' and 'growth rate' in the literature was identified and the subtle effect of new horn growth from the coronary band on distal hoof wall repair was acknowledged and investigated. The results have been published (Reilly *et al.* 1996, 1998a, 1998c).

3. Growth of wall horn between the left fore feet of pairs of ponies at the midline dead centre (MDC) sampling site was used as an original means of identifying that horn which had grown under the experimental conditions and of ensuring that only that horn was used for subsequent comparative testing.

4. Within an equivocal literature data base, this trial contributed controlled data to the belief that biotin *does* increase hoof horn growth and growth rate as a 'supraoptimal response (Cuddeford 1991) in ponies with normal feet, and agrees with the findings of Buffa *et al.* (1992), with horses, which were also from a controlled trial.

5. The trial uniquely identified that there was no difference between feet for hoof growth and growth rate and that the two older animals in the trial had lower hoof growth than the other ponies.

6. A novel method of dividing the hoof wall *stratum medium* at the MDC, using a grid, resulted in a unique quantification of Tubule Density (TD). Previous workers had not taken this approach and had either quoted TDs for the whole or part of the hoof wall depth (HWD) or had simply given descriptive reports of the way tubules were perceived to be distributed in the hoof wall.

7. The new TD-counting method revealed that the hoof wall consisted of four distinct 'zones'. This was new, quantitative, anatomical information which objectively defined previous descriptive reports and it was therefore proposed that the hoof wall may act as a quadri-laminar ply (Reilly *et al.* 1998b).

8. A similar four-zoned pattern of TD with HWD to that in the ponies, was found in a subsequent original study of the front hooves from a random sample of abattoir horses. It was therefore proposed that the four-zoned arrangement of TD within the MDC of the hoof wall is an equine pattern (Reilly *et al.* 1998b)

9. Biotin supplementation caused an increase in TD in only Zone 4 of the SM for the ponies in this trial. Only Kempson *et al* (1989), with pigs, and Dittrich *et al* (1994), with horses, had previously reported an increase in whole-wall mean TD with biotin supplementation .The work in this thesis represents the first time such a site specific change in TD with biotin supplementation has been reported.

10. The public domaine software package NIH Image was specifically adapted to the task of morphometric measurement of histological slides of the hoof wall. Using this method, biotin supplementation was found to have caused a decrease in mean marrow size of Zone 1 tubules in treatment compared to control animals in the two younger pairs of ponies. This was the first time such a site specific and absolute size change with biotin supplementation had been quantitatively reported.

11. The imaging method also allowed the tubular to intertubular horn area fraction ratio to be quantified. In the past this ratio has been estimated by other workers to be 1:3, wheras in fact it was found to be 1:1.

12. The work in this thesis was unique in its field in that it measured the moisture content of pony hoof horn in three different, and controlled, states: 'fresh', 'fully hydrated' and 'dry'.

13. It also used a novel method to determine material stiffness from 3-point bending using sample beams.

14. The work uniquely identified that biotin had no effect on moisture content in any of these states, nor on the material stiffness in the fresh and dry states.

15. Biotin treated horn was found to show a significant increase in stiffness in the fully hydrated state only. This was a unique finding.

16. Evidence has been given in this thesis to support the notion of a nutrition hoof horn axis for biotin. In a further study to assess the effects of another nutrient (Reilly et al 1998c) unique information was given about the lipid content of hoof horn and how it, too, responded to oral supplementation. This was the first time such direct evidence of such a nutritional effect with lipid, at the level of the hoof, had been demonstrated.

17. Finite element analysis represents a means of modelling hoof wall responses to given conditions. As a start to building this area of expertise, the gross hoof capsule of a donkey in static loading was modelled for the first time and this was followed by a unique microscopic FEA model of the outer aspect of the donkey hoof wall *stratum medium* . The results have been published (Newlyn *et al* 1998 and Newlyn *et al* 1999).

CHAPTER EIGHT

Future research directions

Based on the data presented in this thesis and the implications of it, which were discussed in individual Chapter discussions and in Chapter 6, a summary of suggested future research directions is now given:

1 . Repeat trials with the same supplements (i.e. biotin and EPOM) should be conducted in order to assess whether the responses found in the work for this thesis are reproducible, in ponies, under differing conditions.

2 . Repeat trials for horses and for other hooved animals are recommended in order to assess whether the responses found in this trial are similar across the hooved species.

3 . All further trials should be conducted in a double-blind, placebo-controlled, cross-over fashion. They should run for such time as to allow full hoof capsule renewal and involve sufficient numbers of animals of disparate ages and bodyweights to allow the effects of these variables on measured parameters within the hoof wall to be assessed.

4 . The cellular and molecular bases of the growth and growth rate responses found in this thesis for biotin and EPOM supplementation should be pursued in order to increase our understanding of their potential use as medical intervention in hoof horn management.

5. Further work into dose rate responses with biotin and EPOM should be conducted in order to establish the optimal levels of supplementation for these nutrients.

6 . The effects of different keratogenic nutrients should be assessed using methods 1-5 above, in order to increase our knowledge of the nutritional control of keratinization and in order to increase the potential scope for nutritional intervention in hoof horn management.

7. New methods of quantifying gross hoof horn capsule parameters should be developed to allow objective assessment of the periople, the wall (in other areas aswell as MDC), the hoof wall clipping, the sole, frog, bars and white-line, in order to improve our understanding of normality and increase the number of methods by-which the potential effects of supplementary nutrients can be assessed, at the level of the hoof capsule and, preferably, non-invasively.

8 . Temporal and locational effects on hoof horn parameters within the capsule should be studied in order to increase our understanding of the suitability of potential sites for harvesting samples for subsequent analysis and interpretation of normality.

9. Further studies should be conducted to assess the effect of age of animal on hoof growth at different sites around the hoof wall, as this information is currently not known. A knowledge of hoof growth and hoof growth rates between experimental animals is required to ensure like sampling between animals.

10. In future work mechanical and moisture-content measurements should be conducted *within* the different zones identified in this thesis. This will allow investigation of whether or not relationships between TD, morphological parameters, moisture content and mechanics exist within these specific areas of the hoof wall and enable the proposal that the hoof wall acts as a quadrilaminar ply to be tested.

11. Further investigations are required to *explain* the mechanism by which TD was increased by biotin supplementation in Zone 4 of the SM. Methods are required to enable the number of papillae that are produced at the corium to be counted and to assess whether these correlate with tubule numbers. The application of stereological techniques to addressing this question would be worthwhile.

12. Further work is recommended to find the cellular and molecular mechanisms which may account for the finding in this thesis that marrow size in Zone 1 can be decreased by biotin supplementation.

Also, since certain hoof horn morphological features, such as tubule cortex area-fraction in Zone 2 and absolute marrow size in Zone 4 were found in this thesis to be significant determinants of mechanical function in bending, at different hydration levels, then further research into the cellular and molecular mechanisms by which these features of the hoof wall are regulated and controlled, at the level of the dermal papilla, would be particularly fruitful.

In vitro methods of sustaining dermal papillae would allow experiments to be conducted to determine the cellular responses at the papilla tip to different nutritional stimuli, and would also allow study of the molecular events associated with such changes.

13. Since biotin was seen to affect the bending stiffness of hoof horn only at the fully hydrated level, but this finding could not be fully explained, further investigations into the ways in which nutritional supplementation may affect the molecular control of keratin protein production and the way moisture is held at the molecular, cellular and structural level within hoof horn are recommended.

14. Finally, FEA and modelling studies are warranted at every level of the structural hierarchy of the hoof wall in order to create a better understanding of the way in which the hoof wall achieves its function and in order to predict the consequences of any change that may be imposed upon it.

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APPENDIX I

Biomimetics, Vol. 4, No. 1, 1996

Tubule Density in Equine Hoof Horn

J. D. Reilly[1,2], D. F. Cottrell[1], R. J. Martin[1], and D. Cuddeford[1]

The number of tubules per square millimeter of equine hoof horn tissue was quantified in samples taken from the left forefeet of eight ponies which had been matched for age, sex, and weight and reared under controlled conditions. The objective assessment of the arrangement of tubules within the stratum medium of the hoof wall has been overlooked in the past. There is a distinct zonation of tubule density that may represent an equine pattern.

KEY WORDS: equine; hoof horn; tubule density; composite; biomimetics.

INTRODUCTION

Hoof horn is a composite material at two levels: first, as tubular and intertubular horn at light-microscopic magnifications and, second, as the filamentous protein keratin, suspended in a matrix within keratinocytes, when viewed with the electron microscope (Fig. 1).

The fine function of the hoof capsule is likely to be dictated by contributions from

- the biochemistry of fibril and matrix relationships,
- the volume fractions of intracellular components,
- the chemistry of intercellular bonding,
- the volume fractions of intercellular components,
- the direction of fibril winding in tubular and intertubular horn,
- the orientation of the cellular axes in tubular and intertubular horn.

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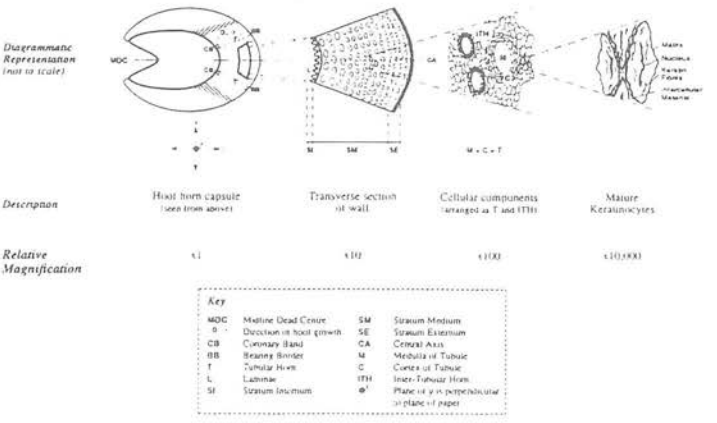


Fig. 1. Components of the hoof wall.

- cell sizes,
- tubule density,
- tubule size, and
- the volume fractions and relative proportions of tubule components and intertubular horn.

Hoof wall function will also be modulated by moisture content (Bertram and Gosline, 1987).

The existence of tubular and intertubular horn in the hooves of cattle and horses has been known for some time (Nickel, 1938, 1939; Wilkens, 1964), although in the earlier literature the tubules were, confusingly, referred to as "horn filaments" (Fleming, 1871). These tubules are assumed to be continuous, to be in the same plane as hoof wall growth (D with dashed arrow in Fig. 1), and to run down the length of the wall from the coronary band (CB in Fig. 1) to the bearing border (BB in Fig. 1) at the ground surface (Pollitt, 1995). They are assumed to be parallel to the wall (Greenough *et al.*, 1972). A broadly similar arrangement exists for hoof horn in pigs (Geyer and Tagwerker, 1986), sheep (Rosskopf and Geyer, 1987) and cattle (Greenough *et al.*, 1972), although there are differences in anatomical detail. Each of these species has different physical demands made on their hooves as a function of body weight, size, and environment. The horse has the greatest physical demand made upon it in terms of athleticism. In this regard it represents a pinnacle of evolution and selection. Since the hoof wall plays a major role in damping the concussive forces of

locomotion (Dyhre-Poulson *et al.*, 1994) and its arrangement as tubular and intertubular horn is believed to be important in stress transfer and resilience (Nickel, 1938, 1939; Wilkens, 1964), it is, perhaps no coincidence that equine hoof horn has a very distinct organization of tubular and intertubular horn. Bertram and Gosline (1986) suggested that the arrangement of component horn tubules will have important consequences on the way in which forces are distributed within the equine hoof wall. Thus it will affect the hoof's ability to "transmit the forces of locomotion painlessly to and from the axial skeleton" (Reilly and Kempson, 1992).

Schaller *et al.* (1973) and Schaller (1992) recognized three distinct areas of the equine hoof wall: stratum externum (SE), stratum medium (SM), and stratum internum (SI) (Fig. 1). The bulk of the wall is made up of the stratum medium, the main supportive structure (Pollitt, 1992), and is the subject of this paper. Note also that this paper deals with the central midline axis of the hoof [midline dead center (MDC) in Fig. 1] only, as the proportions and components of the wall change at other anatomical sites. (Reilly, unpublished observations).

Nickel (1938, 1939) and Wilkens (1964) described the differences in tubule size, orientation of component cells, and fiber winding within tubular and intertubular cells for different parts of the hoof wall in the horse and cow. However, neither quantified the way these tubules were distributed and Leach (1980) and Stump (1967) gave further unquantified reports of the arrangement of tubules within the equine hoof wall. Tubule densities for hoof tissue from different species are given in Table I. None of these authors have detailed the specific methods by which tubule densities were calculated in each case.

It is believed that "horn quality" is determined to a large degree by the number of tubules per unit area in the cow (Politiek *et al.*, 1986) and in the pig (Geyer and Tagwerker, 1986). "Hardness" of horn is believed to be directly related to the number of tubules per unit area in cows (Gunther *et al.*, 1983)

Table I. Hoof Wall Tubule Density Data for Different Species

Animal	Tubule density (tubules/mm^2)	Method	Reference
Horse	30	Light microscope	Leach (1980)
Horse	8–14	Light microscope	Bucher (1987)
Cattle	80	Unknown	Vermunt and Greenough (1995)
Sheep	99	Light microscope	Rosskopf and Geyer (1987)
Pig	80–100	Light microscope	Geyer and Tagwerker (1986)
Pig	99	Electron microscope	Kempson *et al.* (1989)

and in pigs (Geyer and Tagwerker, 1986). However, there is a lack of objective definition of "horn quality" (Reilly, 1995) and few established relationships reported between mechanical properties and structural features of the hoof.

There is now a need to employ objective methodologies to establish a common basis for reporting the features of hoof horn from different species and to investigate the relationships between anatomical features of hooves and mechanical properties.

The aim of this paper is to give a method by which tubule density may be obtained in hoof horn samples from the stratum medium of hoof wall from equids. It also provides an objective tool, and a common language, for investigating the composite properties of hoof horn from different species. This will help reveal aspects of the structure and function of hoof horn, which is designed to cope with different kinds of natural impact and thus help in biomimetic design as Vincent (1992) has envisaged.

MATERIALS AND METHODS

Trial Design

Four pairs of ponies were matched for sex, age, and weight (Table II). They were fed a commercial high-fiber pony cube preparation as a common basal diet for a period of 12 weeks. After this time one of each pair of horses was randomly allocated to continue with the basal or "control" diet, or to start a different "treatment" diet. This was exactly the same as the basal diet except for the addition of a certain vitamin under test. The trial continued for a further 5 months. The extent of new horn growth for all individuals was recorded by monitoring the descent of a branded reference mark on the hoof wall (X in Fig. 2) with respect to the CB. The animals were then humanely killed at an abattoir.

Table II. Animals Used in the Trial

Pair No.	Description	Weight (kg)	Age (yr)	Sex
1				
Pony 1	Chestnut Welsh type	245	2	Mare
Pony 5	Dun Welsh type	270	2	Mare
2				
Pony 2	Bay Welsh type	257	7	Gelding
Pony 6	Bay Welsh type	235	6	Gelding
3				
Pony 3	Chestnut Shetland	204	8	Mare
Pony 7	Black Shetland	212	8	Mare
4				Mare
Pony 4	Black Shetland	170	12	
Pony 8	Black Shetland	160	14	Mare

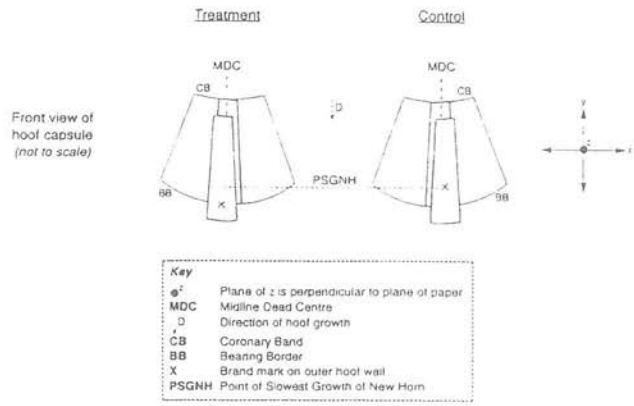


Fig. 2. Sampling of hoof horn for quantitative tests.

Distal limbs were disarticulated at the fetlock after labeling. The hooves were wrapped in a double layer of cling film and transported to the laboratory on ice.

Dissection and Definition of Sample Sites

The front left foot from each pair of animals (treatment and control) was used to give hoof samples for quantitative tests. In the *x* plane, the site of sampling was the midline dead center (MDC) stratum medium of the hoof wall (Fig. 2). The MDC was marked with chalk on the outer surface of the hoof wall in the *y* plane. The samples were then cut from the hoof wall such that they were symmetrical about the MDC in the *x* plane.

Treatment and control hoof horn grew at different rates during the trial. Thus, the point of slowest growth of new horn (PSGNH; Fig. 2) that was reached between the two individuals of a pair determined the distal boundary for sampling for that pair (Fig. 2). Then, in a proximal direction and in the *y* plane, three sample blocks were cut for mechanical testing and one for histological preparation for histomorphometry. The hoof samples therefore came from different points, in the *y* plane, on the hoof wall between pairs but at the same point within pairs.

Histology/Determination of Tubule Density

Horizontal sections, in the *z* plane, of 10-μm thickness were cut from each block on a cryotome and stained in alcian blue/periodic acid Schiff, dehydrated, and mounted in DPX with a coverslip. The stain is preferentially taken up by

intercellar material and by the tubule medullae. Photographs were taken of each section and of a calibration graticule, and both were enlarged photographically to A4 proportions for ease of working.

Consistent alignment of the grid between sample prints was achieved by defining a central axis (CA) for each section:

The stratum externum (SE; Figs. 1 and 3) has a curved exterior in the x plane; the stratum internum (SI; Figs. 1 and 3) is made up of a "fan" of radially oriented laminae (L; Figs. 1 and 3). The SI has a curvature different from that of the SE and the forward projections of the laminae do not always form a smooth line. The CA was given by a line which was in the center of the histological section and which ran from the curvature of the SI to meet the SE at a right angle (C.A. in Fig. 3). The CA was consistent with the MDC (see Figs. 1 and 3) since the CA represented the center of a section that had been cut from the hoof wall symmetrically about the MDC.

Since a concentration of stain was taken up by tubule medullae, this defined the location of any given tubule within the SM of the hoof wall (M in Fig. 3). A grid of parallel horizontal lines, at 0.5 mm intervals, was then superimposed on the A4 photographic print such that its lines were perpendicular to the CA. The area between grid lines was referred to as a "cell." Alignment of the grid in the z plane was such that the first cell for counting within was the one in which the full width of field was covered by stratum medium ("first cell" in Fig. 3). The final cell that contributed to the count in the z plane was that which gave a full width of field without being interrupted by any part of a lamina (L) from the SI ("last cell" in Fig. 3).

In this way tubule point-counting in relation to the area between the grid lines, by depth into the hoof wall, was facilitated. The method employed for counting medullae within a cell is similar to that used in a hematocytometer. Bush (1975) explains that not all workers use the same convention and so his recommendations are followed. The protocol used is summarized in Fig. 4. This method gave the initial tubule density (ITD), which was the number of tubule medullae that were counted per unit area of cell within the grid.

Tubule density (TD) was the ITD converted to give the equivalent number of tubule medullae per unit area of hoof horn tissue proper by using a conversion factor (CF) derived from the scaled photographs.

To normalize for differences in hoof wall depth between individuals, tubule density was expressed by percentage hoof wall depth (%HWD):

$$\%\text{HWD per cell} = \frac{100\%}{\text{Total number of cells from "first cell" to "last cell"}}$$

This meant that for each animal the fixed cell dimensions represented a different percentage of the wall depth for that animal.

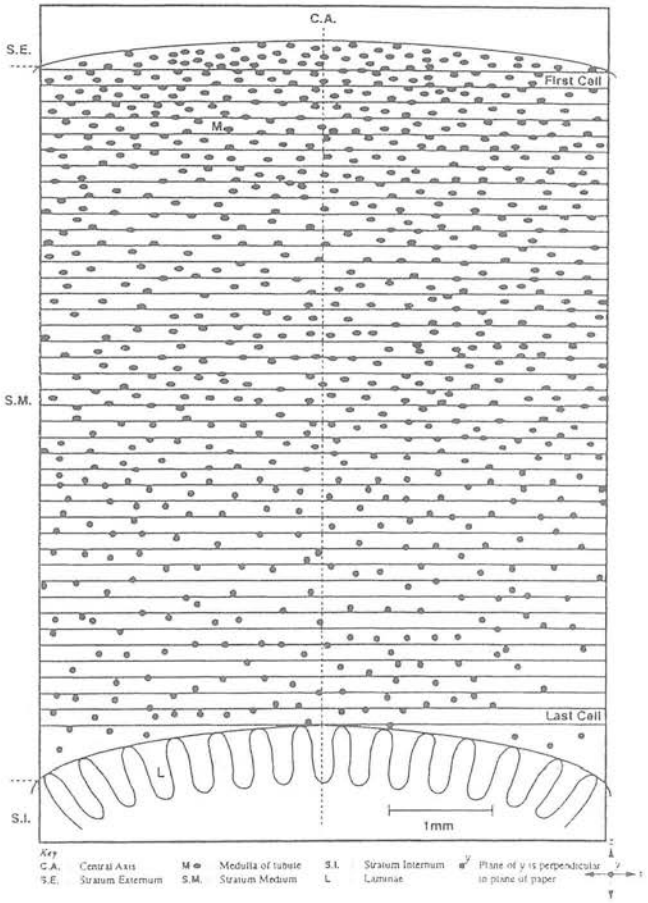
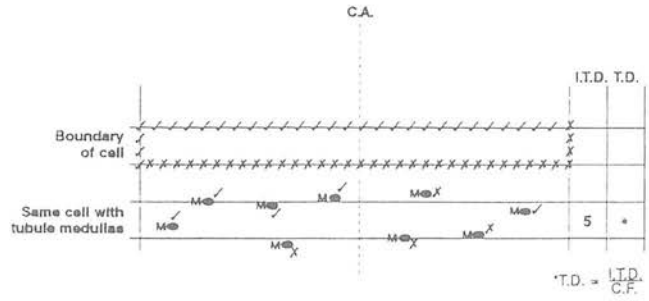


Fig. 3. Divison of the stratum medium of the hoof wall for tubule counting.



Key
C.A. : Central Axis
✓ : Counted
✗ : Not counted
M⊕ : ABPAS stained tubule medulla
I.T.D. : Initial Tubule Density
C.F. : Conversion Factor
T.D. : Tubule Density

Fig. 4. Tubule counting protocol.

Data analysis and graphical and tabular presentations were completed using Minitab and Statview statistical packages.

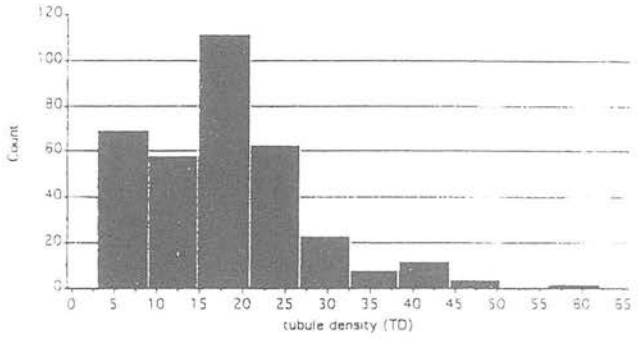
RESULTS

A frequency distribution histogram of the tubule density data is given (Fig. 5). The distribution is skewed to the right so that the mean and median are not the same. Using a normal probability plot (Minitab Inc.), the hypothesis for normality was rejected at $P < 0.1$. The tubule density data set therefore has a nonnormal distribution.

Figure 6 shows a sq. root transformation of Fig. 5 and the mean and median are the same. The probability that the relationship has a normal distribution, using the same method, is $P > 0.99$.

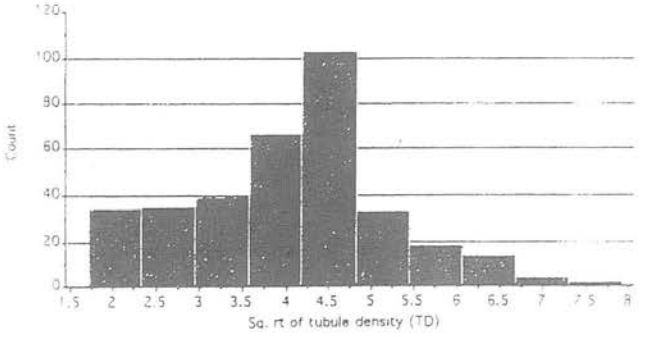
The results for tubule density (TD) by percentage hoof wall depth (%HWD) are presented as a scattergram (Fig. 7) showing two areas of changing tubule density (Zones 1 and 3) and two areas of relatively unchanging tubule density (Zones 2 and 4).

Dividing the transformed tubule density data set (Fig. 6) by conventional



Count	Mean	Median	Std. Dev	Kurtosis
344.00	17.53	17.00	9.43	1.44

Fig. 5. Frequency distribution of tubule density data



Count	Mean	Median	Std. Dev	Kurtosis
344.00	4.03	4.03	1.14	-0.15

Fig. 6. Frequency distribution of transformed tubule density data

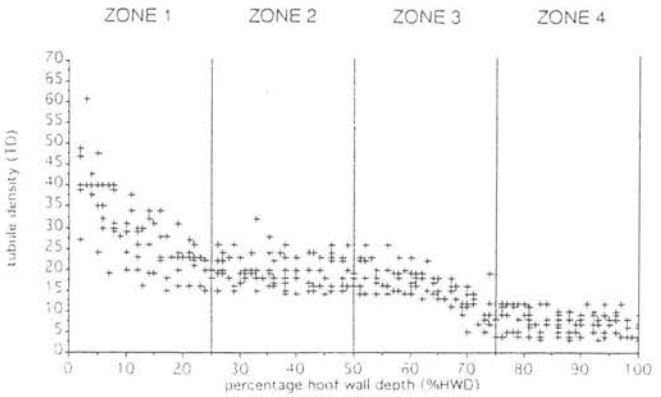


Fig. 7. Pony tubule density (TD) by percentage hoof wall depth (%HWD) to show tubule density zonation.

use of ±1 and 2 standard deviations about the mean gives divisions at 1.75, 2.89, 4.03, 5.17, and 6.31 $\sqrt{\text{tubules/mm}^2}$. These five divisions correspond to TDs on the y axis in Fig. 7 of 3.0, 8.35, 16.24, 26.7, and 39.8 tubules/mm^2, respectively. To find the corresponding points on the x axis in Fig. 7 (i.e., %HWD), a simple regression equation was used to describe the relationship between $\sqrt{\text{TD}}$ and %HWD. The equation is as follows:

$$\%\text{HWD} = 141 - 2.3\sqrt{\text{TD}}$$

By substituting the above values of $\sqrt{\text{TD}}$ at standard deviation divisions for x in the above equation, values for y (%HWD) were given as 76.55, 51.13, and 25.71%, which splits the SM into four zones.

These values occur at approximately 25% intervals and appear to correspond to where the inflections of a curvilinear "line of best fit" would occur in Fig. 7.

Thus the following estimated zones are proposed for the division of the SM of these ponies on the basis of approximate TD:

Zone	%HWD	TD (tubules/mm^2)
1	0–25	>27
2	25–50	16–27
3	50–75	8–16
4	75–100	<8

DISCUSSION

Division of the stratum medium of the hoof wall of these ponies into four separate zones on the basis of tubule density is proposed.

This objective division of the stratum medium of the wall according to tubule density correlates well with previous descriptive reports. Nickel (1938, 1939) and Wilkens (1964) described three layers: an inner, a middle, and an outer layer. These are equivalent to Zones 1, 2, and 4 described in this paper. Stump (1967) agreed with these descriptions but also reported an "intermediate layer" between Nickel's (1938, 1939) and Wilkens' (1964) inner and middle layers. This intermediate layer would correspond to Zone 3 proposed here.

The results from the selected and controlled population of ponies in this work corroborates the descriptions of the wall tubules from previous workers (who used samples from a wide variety of horses). This implies that the zonation described is a generalized equid pattern.

Another population of tubules is reported to exist between what is described as Zone 4 here and the SI. These have been described by other workers such as Leach (1980), Bolliger (1991), Bragulla *et al.* (1994), and Budras and Huskamp (1994). This method does not describe them as a separate entity either because their density has made little impact on the data derived from the "last cell" in the grid or because they have not been counted as a result of the discrepancy in alignment between the straight edges of the grid and the curvature of the SI.

Similarly, the degree of curvature of the SE surface will affect the outer zone tubule density readings. The method may therefore underestimate the true mean tubule density of the SM by not detecting the full contribution that may be made by Zone 1. Indeed, it may have omitted the possibility that the outermost tubules that occur in large numbers per square millimeter of horn tissue may represent another population of tubules in the outer SM.

The tubule density data set in this work did not have a normal distribution. The "mean" figure for tubule density derived from the untransformed data is 17.5/sq mm^2 (Fig. 5) and is therefore in broad agreement with Bucher (1987). Transformation of the data (Fig. 6) shows that this is, however, an overestimate for the tubules counted. The true mean tubule density figure for the data is $(4.03)^2$, which is 16.2/mm^2. In addition, from the distribution of the density of tubules (Fig. 7) and the zonation proposed above, care must be also taken to take account of possible sampling effects in the generation of tubule density data sets. These factors should be borne in mind when interpreting tubule density data that are quoted between workers.

Leach (1980) postulated that during weight bearing much of the ground reaction force is transmitted proximally up the wall (Thomason *et al.*, 1992).

The high tubule density in Zone 1 may allow stress to be concentrated in this part of the hoof wall as a function of its load-bearing properties. The rapid decline in tubule density in Zone 1 and the step-like pattern of tubule density found in Zones 1–4 may be a mechanism for the smooth transfer of stresses from the SM to the SI and thus to the axial skeleton to which the SI is attached. Tubule density gradation across the wall could therefore be a mechanism for smooth energy transfer which would act in concert with stiffness changes that were mediated by changing hydration levels as suggested by previous workers (Leach, 1980; Leach and Zoerb, 1983; Bertram and Gosline, 1987; Bertram *et al.*, 1992).

Since energy is absorbed by separating two phases of a composite as a growing crack approaches (Cook and Gorden, 1964; cited by Bertram and Gosline, 1987), the tubule/intertubular interface in the composite structure of the hoof is acting as a "crack stopper." The higher tubule densities in the outer zones will afford a protective mechanism for ensuring that cracks do not propogate to the inner zones, which are nearer sensitive structures within the foot. Such mechanisms will be modulated by water content within the zones of the hoof wall and by other features of the hoof's morphometry and chemistry such as those governing the strength of intercellular bonding. However, zonation of the stratum medium, according to tubule density, allows the hoof wall to act as a laminated structure. The ability to shed an outer layer, or zone, or hoof wall that has contained a crack, as is often seen in horses (Reilly personal observations), would then be a "fail-safe" mechanism that, paradoxically, protects the hoof capsule from catastrophic failure. Provided that the lost material is close to the BB, it can be replaced relatively quickly due to the mechanism of hoof wall growth. Other types of hoof wall cracking that are seen may also be governed in part by the strength of the interface between zones within the hoof wall. Thus, this arrangement of tubules within the hoof confers upon it the design advantages of a laminated composite.

Tubule density can be used as a defining parameter for the location of a site of interest, or of comparison, within the MDC SM. It also gives a method for comparing the distribution of tubules within the SM of the hooves of other species: If similar patterns of zonation exist, or do not exist, this will indicate methods of adaption.

With more consideration of zonation of tubules within the equine hoof wall, and by defining the distinct properties of these zones, our understanding of hoof wall function will increase. The existence of distinct zones within the hoof wall will have important implications for the manipulation and management of the hooves of horses and for biomimetic designs destined to mimic the situations with which they deal.

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APPENDIX II

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Effect of supplementary dietary biotin on hoof growth and hoof growth rate in ponies: a controlled trial

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Keywords: horse; biotin; hoof; growth; growth rate; pony

Summary

The effect of dietary biotin supplementation, at a dose rate of 0.12 mg/kg bwt, on growth and growth rate of the hooves of 8 match-paired ponies was investigated in a controlled feeding trial. Treatment animals had a mean hoof growth at the midline dead centre of the hoof capsule of 35.34 mm after 5 months of biotin supplementation compared to control animals 30.69 mm ($P<0.05$). Comparison of regression analysis also showed that biotin supplementation produced a significantly higher ($P<0.02$) growth rate of hoof horn in this trial. Treatment animals had a 15% higher growth rate of hoof horn and 15% more hoof growth at the midline dead centre, after 5 months of biotin supplementation compared to control ponies. No differences were found between feet for growth of horn, but the older animals in the trial had significantly lower hoof growth ($P<0.05$) than the remaining ponies.

Introduction

Hoof horn 'development', 'structure' and 'integrity' have all been reported to be influenced by dietary changes (Comben *et al.* 1984; Kempson 1987, 1990). However, these were individual case reports that were uncontrolled (Slater and Hood 1997). The methods used for assessment were subjective and no data were provided to support the claims made. Presumably, the reason subjective assessment, as opposed to objective measurement, was used is that there are few features of the hoof which, at first sight, appear suitable for quantitative definition. Objective measurement of defined features of the hoof capsule therefore becomes the challenge in this field of study (Reilly 1995).

Hardness (Buffa *et al.* 1992), tensile strength (Geyer and Schulze 1994), tubule density (Reilly *et al.* 1996), modulus of elasticity (Landeau *et al.* 1983), work of fracture (Bertram and Gosline 1986, 1987), moisture content (Leach 1980), and hoof horn growth (Buffa *et al.* 1992) are examples of quantitative variables that can be measured. They are useful because they give an objective measure of the anatomical, material and physiological characteristics of hoof horn, an understanding of which enables factors governing its function to be unravelled.

The importance of hoof horn growth was recognised by Caulton Reeks (1906) who mentioned that the slow hoof growth found, even in the normal horse, is frustrating for the veterinarian. Geyer and Schulze (1994) expressed the effect of growth rate on the 'renewal time' of the hoof capsule, while Butler and Hintz (1977) acknowledged that the 'rate of hoof growth is of vital importance since it often affects the usefulness of the horse'.

Manipulation of hoof horn growth rate can have practical implications for veterinary treatment and farriery in terms of growing out a lesion, or for producing sufficient horn for nailing in to. A knowledge of hoof horn growth is also an important research prerequisite for meaningful sampling of like material for comparative testing (Reilly *et al.* 1996).

However, there is misuse of the terms 'growth' and 'growth rate'. Growth rate has been used where growth has been calculated or observed (Butler and Hintz 1977; Guthrie and Stoker 1990; Buffa *et al.* 1992), and the 2 terms have been used synonymously. They are different variables.

Hoof growth gives an indication of the total amount of material that has been produced at the end of a given period with units of cm or mm (Dittrich *et al.* 1994; Graham *et al.* 1994; Ott and Johnson 1995).

Hoof growth rate is a time dependant variable and gives an indication of the pace at which that material has been accumulated and, therefore, has units of cm/month or mm/day. (Geyer and Schulze 1994; Josseck *et al.* 1995). The same hoof growth can be achieved by variable periodic growth rates. Hoof growth rate can be given by the slope of a growth curve or by rates that have been calculated on a periodic basis, with growth up to that time being divided by the time relating to that period (Geyer and Schulze 1994; Josseck *et al.* 1995). Reported growth rates for equine hoof horn are given in Table 1.

There are few substantiated reports of factors affecting hoof growth rates. Shannon and Butler (1979), and Geyer and Schulze (1994) suggested seasonal differences, with higher growth rates in spring and summer and decreased growth rates in the winter. Geyer and Schulze (1994) found breed differences. Graham *et al.* (1994) and Ott and Johnson (1995) suggested sex differences with male rates higher than female rates in younger horses, although Butler and Hintz (1977) found no differences in growth between the sexes. Butler and Hintz (1977) also concluded that the level of protein intake did not affect hoof growth, but that total dietary intake did, with young ponies on *ad libitum* feed achieving more growth than those on restricted intakes. In studies investigating

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TABLE 1: Equine hoof growth rates (reported measurements converted to daily rates)

Growth rate (mm per day)	Author
0.19–0.28	Glade and Salzman (1985)
0.200	Kainer (1987)
0.208	Caulton Reeks (1906)
0.248 (left fore), 0.257 (right hind) (Lipizzaners)	Josseck (1991)
0.248 (biotin) 0.25 (control) (Lipizzaners)	Josseck *et al.* (1995)
0.273 (control) 0.333 (biotin)	Buffa *et al.* (1992)
0.286 (Shires & Belgians) 0.143-0.179 (Icelandics)	Geyer and Schulze (1994)
0.287	Knezevic (1959)
0.384 (fed ad lib), 0.254 (limited feed) (8 months old)	Butler and Hintz (1977)
0.445 (diet including proteinate) (yearlings)	Ott and Johnson (1995)
0.427 (control) (yearlings)	Ott and Johnson (1995)
0.4375 (yearlings)	Graham *et al.* (1994)
0.48 (control) 0.42 (biotin) (yearlings)	Dittrich *et al.* (1994)

hoof growth it is therefore important to control for these factors.

The features of the hoof capsule vary at different sites both radially and proximodistally. Whether growth of horn at different radial sites around the hoof capsule takes place at different rates is unclear: Caulton Reeks (1906) and Kainer (1987) suggest that this growth is even, radially, around the coronary band, whereas Geyer and Schulze (1994) reported that the 'palmar/plantar' part of the wall tended to have a lower growth rate in comparison to the dorsal and lateral parts. Josseck *et al.* (1995) also found differences in growth rate between midline, lateral sidewall and palmar/plantar sites.

Therefore, the precise definition of measurement site is important in order to give results a context and to allow repetition of study.

Butler (1976) and Scott and Butler (1980) found that hind hooves grew faster than fore and it can sometimes be unclear which measurement(s) from which site(s) on which feet are contributing to the data presented.

Butler (1976) pooled data from one site on all 4 feet. Graham *et al.* (1994) used a midline site and pooled data from the forefeet only. Geyer and Schulze (1994) used 3 measurement points (dorsal, lateral and palmar) and pooled these data from all 4 hooves in the first year of study and only from the left fore (LF) and right hind (RH) thereafter. Josseck *et al.* (1995) used the same 3 sites as Geyer and Schulze (1994) and used data from LF and RH only. In addition to defining the site(s) for measurement, it is important to state which feet are contributing to the data set.

Apart from objective measurement, Cuddeford (1991), Reilly (1995) and Slater and Hood (1997) have all called for controlled experimentation in this field of study. May (1989) concluded that 'most of the claimed treatments for poor quality hoof horn were empirical and anecdotal'. This remains true and heed must be taken of the recent call for more rigour in setting up experiments involving equids which aim to assess the effects of a therapy. If this is not done then 'therapy becomes lore; medical myths have arisen this way' (Rossdale 1997). This is in danger of becoming the case in this field of study. Unfounded attempts at therapy for hoof horn problems perpetuate medical myths.

More recently controlled studies have been carried out by Buffa *et al.* (1992); Dittrich *et al.* (1994); Josseck *et al.* (1995) and Zenker *et al.* (1995). Coincidentally all these experiments investigated the effects of supplementing dietary biotin. The background leading to the use of biotin has been covered before

TABLE 2: Details of ponies in the trial, feed intake and biotin intake

Pair	Animal	Treatment (T) or Control (C)	Description	Weight (kg)	Age (yrs)	Sex	Mean pair bodyweight (kg)	Fresh weight feed per day (split into 2 feeds) (kg)	Total biotin intake basal + supplemented (mg)	Biotin dose rate (mg/kg bwt)
1	Pony 1	C	Chestnut welsh type	245	2	Mare	257.5	3.86	0.386 + 0 = 0.386	0.00158
	Pony 5	T	Dun welsh type	270	2	Mare			0.386 + 30.88 + 31.266	0.1158
2	Pony 2	C	Bay welsh type	257	7	Gelding	246	3.69	0.369 + 0 = 0.369	0.00144
	Pony 6	T	Bay welsh type	235	6	Gelding			0.369 + 29.52 = 29.889	0.1272
3	Pony 3	C	Chestnut shetland	204	8	Mare	208	3.12	0.312 + 0= 0.312	0.00153
	Pony 7	T	Black shetland	212	8	Mare			0.312 + 24.96 = 25.272	0.1192
4	Pony 4	C	Black shetland	170	12	Mare	165	2.48	0.248 + 0 = 0.248	0.00146
	Pony 8	T	Black shetland	160	14	Mare			0.248 + 19.84 = 20.088	0.1256

TABLE 3: Nutritional specification of basal diet

Component	Proportion in diet as fed
Dry matter	86%
Energy	9.6 MJ/kg DM
Crude Protein	10%
Oil	2.75%
Fibre	20%
Ash	8.5%
Vitamin A	5800 iu
Vitamin D3	1000 iu
Vitamin E	9 iu
Vitamin B1	1 mg/kg
Vitamin B2	3.62 mg/kg
Nicotinic Acid	25 mg/kg
Pantothenic Acid	10 mg/kg
Biotin	100 (µg/kg
Vitamin B12	18 (µg/kg
Vitamin K	1 mg/kg
Manganese	11 mg/kg
Zinc	50 mg/kg
Iron	33 mg/kg
Cobalt	0.25 mg/kg
Iodine	0.4 mg/kg
Calcium	1.2%
Phosphorus	0.5%
Copper	13 mg/kg
Selenium	0.2 mg/kg
Molybdenum	1.0 mg/kg

(Buffa *et al.* 1992; Reilly 1995).

Biotin is a water soluble B group vitamin and is an essential co-factor in glucose and fat metabolism. It can have profound effects on other pathways by its influence on many other intermediaries (Whitehead 1981). It is essential for growth (Tagwerker 1983) and maintenance of epidermal tissues (Geyer and Tagwerker 1984). It is readily absorbed after oral administration and its plasma kinetics are well described (Josseck 1991; Lindner *et al.*1992; Josseck *et al.*1995). It is not stored in the body (Buffa *et al.* 1992) and is safe to feed at high doses as it is excreted via the kidneys in excess. Its lack of toxicity is a major reason for its use in equine feeding trials aimed at assessing the effects of dietary manipulation on hoof horn changes.

Even with more recent controlled studies, whether or not dietary biotin supplementation has an effect on the growth of hoof horn has had equivocal results: Bains (1985) and Buffa *et al.* (1992) found that biotin supplementation increased hoof growth, Geyer and Schulze (1994) and Josseck *et al.* (1995) found no difference in growth rate following supplementation; and Dittrich *et al.* (1994) reported a decrease in hoof growth.

Some of the differences in results from previous workers may be attributable to the different dosages used. Buffa *et al.* (1992) gave 7.5 mg and 15 mg of biotin to horses of, presumably, differing bodyweights. Geyer and Schulze (1994) gave 20 mg to Warmbloods as did Josseck *et al.* (1995) to Lippizanners. Dittrich *et al.* (1994) gave 10, 20 and 40 mg, as single doses to horses for which bodyweights and ages were not reported. Assuming a 500 kg bodyweight horse, these equate to dose rates of 0.015 mg/kg bwt for 7.5 mg, 0.03 mg/kg bwt for 15 mg, 0.04 mg/kg bwt for 20 mg and 0.08 mg/kg bwt for 40 mg.

Therefore hoof horn growth studies can be confounded by factors such as age, sex and breed of horse, site of reading on the hoof capsule, time of year and dosage given. These factors must be taken into account during experimental design.

The aim of this experiment was to investigate the effect of dietary biotin supplementation at one dose rate on hoof growth and growth rate at a specified site on the hoof capsule of all 4 feet of ponies used in a controlled matched-pair designed feeding trial.

Materials and methods

Trial design

Four pairs of ponies were matched for sex, age, weight, size and breed type (Table 2). Their previous history was largely unknown but they were selected in a vetting based on general signs of health and lack of obvious hoof capsular defects. Prior to purchase, potential pairs were aged from their dentition by 2 different veterinary surgeons. All animals were treated with anthelmintics on arrival, had their teeth rasped and their feet trimmed for mediolateral balance. No abnormalities were revealed by individual oral glucose absorption tests or by routine haematology and biochemistry. All 8 animals were then loose-housed in a barn on wheat straw bedding with access to *ad lib* water. They were fed a commercial high fibre pony cube diet which contained 100 µg/kg of biotin. The details of the cube diet are given in Table 3. This was fed, from individual feed mangers, as a common basal diet for a period of 12 weeks prior to the supplementation experiment beginning, and gave a period during which controlled basal hoof growth could take place. No further supplement, nor hay, was fed although each of the animals had equal access to eat its bedding. The total daily ration allocated to each individual was calculated on the basis of 0.015 kg food fed/kg bwt. In order to control for the effect of total energy intake on hoof growth (Butler and Hintz 1977), each animal within a pair received a ration that was calculated for the mean bodyweight for the pair (Table 2). In this way, energy, protein, mineral and vitamin intakes were controlled.

After 12 weeks, one animal from each pair was allocated randomly to treatment or control diet. The treatment diet was exactly the same as the basal diet except for the addition of 8 mg biotin/kg food as fed. This was also fed on a mean paired weight basis at a rate of 0.015 kg food kg/bwt. The total daily ration was divided to give 2 equal feeds. The absolute amount of biotin fed to each individual within the treatment group varied (Table 2) but the dose rate was consistent at 0.12 mg/kg bwt. Similarly, the absolute amount of biotin fed to control animals maintained on the basal diet varied, but the dose rate to them was 0.0015 mg/kg bwt (Table 2).

There were no problems with acceptance of the food. Biotin supplementation continued for a further 5 months during which period the extent of new hoof growth was measured.

Measurement of hoof growth

New horn streams distally from the level of the coronary band (CB). An assessment of growth was given by measuring the distance to which a hot-branded mark ('X'), made on the hoof wall of each foot of each pony, had descended, at given times during the trial, with respect to a proximal reference point in the region of the coronet. The 'X' mark was made with the flat filed end of a Philips screwdriver which was heated to red hot and placed approximately 1.5 mm into the dorsal hoof wall. The definitive feature used as a reliable proximal fixed point was the reference hairline (RH) at the coronet. This was revealed by turning back the fringe of hair that normally hangs over the CB (Fig 1).

Definition of recording site

Measurements were made at the midline dead centre (MDC) for

TABLE 4: Hoof horn growth data for all ponies by period of trial animal and hoof

Animal and Hoof	RHG_0 (mm)	RHG_1 (mm)	Period 1 G_n (RHG_1-RHG_0) (mm)	RHG_2 (mm)	Period 2 cum. G_n (RHG_2-RHG_0) (mm)	RHG_3 (mm)	Period 3 cum. G_n (RHG_3-RHG_0) (mm)	RHG_4 (mm)	Period 4 cum. G_n (RHG_4-RHG_0) (mm)	RHG_5 (mm)	Period 5 cum. G_n (RHG_5-RHG_0) (mm)
Control animals											
Pony 1 LF	19.0	26.0	7.0	32.0	13.0	39.0	20.0	45.0	26.0	51.0	32.0
RF	16.0	21.5	5.5	24.0	8.0	33.0	17.0	38.5	22.5	47.0	31.0
LH	27.5	30.5	3.0	35.0	7.5	45.0	17.5	52.0	24.5	60.0	32.5
RH	24.0	27.5	3.5	32.0	8.0	44.0	20.0	49.5	25.5	57.0	33.0
Pony 2 LF	16.5	23.0	6.5	29.0	12.5	40.0	23.5	46.5	30.0	50.0	33.5
RF	15.0	18.0	3.0	22.0	7.0	34.0	19.0	39.0	24.0	47.0	32.0
LH	20.0	28.0	8.0	33.0	13.0	45.5	25.5	50.0	30.0	58.0	38.0
RH	25.0	30.0	5.0	34.0	9.0	43.5	18.5	50.0	25.0	54.0	29.0
Pony 3 LF	22.5	31.5	9.0	43.0	20.5	52.5	30.0	55.5	33.0	64.0	41.5
RF	23.5	30.0	6.5	35.0	11.5	46.5	23.0	51.5	28.0	53.0	29.5
LH	28.0	33.0	5.0	39.0	11.0	48.0	20.0	58.0	30.0	63.0	35.0
RH	26.5	34.0	7.5	43.0	16.5	56.0	29.5	60.0	33.5	64.0	37.5
Pony 4 LF	18.0	22.0	4.0	26.5	8.5	36.0	18.0	40.0	22.0	47.5	29.5
RF	12.5	16.0	3.5	20.0	7.5	24.0	11.5	29.5	17.0	35.0	22.5
LH	20.5	22.5	2.0	26.0	5.5	31.0	10.5	34.0	13.5	37.0	16.5
RH	21.0	23.0	2.0	25.0	4.0	31.0	10.0	34.0	13.0	39.0	18.0
Treatment animals											
Pony 5 LF	29.0	34.5	5.5	42.0	13.0	55.5	26.5	61.5	32.5	68.0	39.0
RF	18.5	25.0	6.5	31.0	12.5	42.5	24.0	51.0	32.5	61.0	42.5
LH	26.5	30.5	4.0	36.0	9.5	49.0	22.5	58.0	31.5	65.0	38.5
RH	32.0	37.0	5.0	42.0	10.0	55.5	23.5	63.0	31.0	70.0	38.0
Pony 6 LF	32.5	37.0	4.5	42.0	9.5	53.0	20.5	64.5	32.0	68.5	36.0
RF	16.0	23.0	7.0	31.0	15.0	41.5	25.5	48.0	32.0	52.0	36.0
LH	33.0	38.0	5.0	44.0	11.0	59.5	26.5	64.0	31.0	67.0	34.0
RH	34.0	38.5	4.5	45.0	11.0	55.5	21.5	63.0	29.0	69.0	35.0
Pony 7 LF	23.0	33.0	10.0	41.0	18.0	49.5	26.5	57.0	34.0	65.0	42.0
RF	17.5	24.5	7.0	32.5	15.0	41.5	24.0	48.5	31.0	58.0	40.5
LH	24.0	27.5	3.5	33.5	9.5	43.5	19.5	50.0	26.0	57.0	33.0
RH	24.5	30.5	6.0	39.0	14.5	50.0	25.5	56.5	32.0	66.0	41.5
Pony 8 LF	22.5	27.5	5.0	33.0	10.5	39.0	16.5	44.0	21.5	52.0	29.5
RF	18.0	23.0	5.0	26.0	8.0	29.0	11.0	36.5	18.5	43.0	25.0
LH	21.5	26.0	4.5	30.0	8.5	39.0	17.5	43.0	21.5	51.5	30.0
RH	23.0	26.5	3.5	30.0	7.0	35.5	12.5	43.0	20.0	48.0	25.0

cum: cumulative.

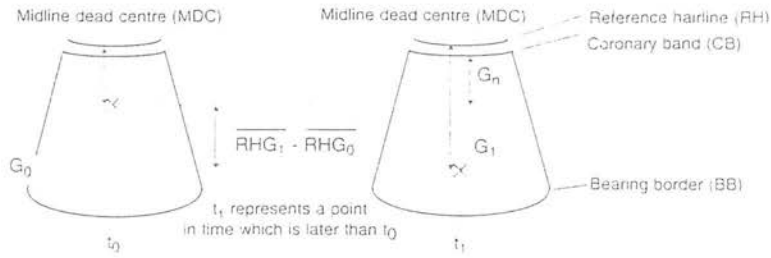
each hoof. The plane of the MDC is that which bisects the foot and is given in Reilly *et al.* (1996). The MDC was given by extending a line that bisected the frog onto the dorsal hoof wall. This then followed the line of the visible dorsal wall hoof tubules to the coronet and was marked with chalk before branding. The initial individual brand marks on the feet of each pony were made below the distal extremity of the periople (XG_0 in Fig 1). Each mark was made at a variable point proximodistally on each hoof, but the distance from it to the RH gave the initial baseline reading for each foot from each animal (RHG_0, column 1, Table 4). The brandmark was not made any higher as the procedure might have damaged periople as well as horn that we were subsequently interested in harvesting for use in other tests. New horn growth from the coronary band, G_n was, therefore, assumed to equal G_1 minus G_0, the difference in measurements from the hairline (Fig 1). The linear distance between the RH and the branded mark was spanned with a pair of dividers. These were then placed against a steel ruler to give a growth reading to the nearest 0.5 mm. Initial growth measurements for the allocated treatment and control groups were made for the last 26 days of the 12 week basal feeding period prior to the start of biotin supplementation. Subsequent growth measurements were made periodically. Period intervals were 32–45 days (Fig 2).

To avoid bias in a nonblinded experiment, data were collected from one group of ponies first and stored, and then data were collected from the other group on the same day. In this way, direct comparison between treatment and control pairs was not allowed during data collection, and analysis of the data did not take place until some months after the experiment had finished.

The data set for hoof growth readings during the course of the trial is given in Table 4.

Statistical analysis

Growth is a continuous variable and would be expected to have a normal distribution. To test this an n-scores probability (Minitab Corp) was plotted for treatment and control data. A correlation of 0.976 for n = 32 (8 horses 4 feet each) was given. This meant that the hypothesis for normal spread of the data could be accepted at a probability of P = 0.01. Therefore, parametric tests; students



Growth is given by: $\overline{RHG_1}$ - $\overline{RHG_0}$ and is equal to the growth from the coronary band, G_n.

Hoof horn growth rate is given by: $\frac{\overline{RHG_1} - \overline{RHG_0}}{t_1 - t_0}$

Fig 1: Measurement of hoof horn growth and hoof horn growth rate at midline dead centre.

t test and analysis of variance were used to assess the data using the Minitab software package.

Results

Hoof horn growth

The cumulative mean growth of horn from the MDC of the feet of all 8 ponies is plotted by period of trial in Figure 2.

The growth of horn for treatment and control groups in the 26 days before supplementation began was compared by *t* test. (Period 1 in Fig 2). There was no significant difference between the 2 groups ($P = 0.62$).

By the end of the trial treatment animals had achieved a mean hoof growth at the MDC of 35.34 mm (s.d. ± 5.63 s.e. ± 1.41) and the control group 30.69 mm (s.d. ± 6.78 s.e. ± 1.70). This difference was significantly different ($P<0.05$) by Student's *t* test.

Therefore, there was a treatment effect on hoof growth after 5 months of biotin supplementation.

Hoof horn growth rate

The slopes of the growth curves (i.e. growth rates), for treatment and control groups were assessed by comparison of regression. The whole data sets contributed to the regression lines.

The regression equation for control growth was:

$$y = 6.5906x - 1.6969$$

The regression equation for treatment growth was:

$$y = 7.6969x - 2.6656$$

The s.d. for control hoof growth rate was 1.263 and for treatment hoof growth rate was 1.107.

Comparison of regression analysis showed that biotin supplementation produced a significantly higher growth rate of hoof horn in this trial ($P<0.02$).

The mean rate of hoof growth over the whole of the trial, by extrapolation from Figure 2, was:

0.164 mm/day for nonsupplemented ponies
and 0.189 mm/day for biotin supplemented ponies

When expressed in percentage terms treatment animals had a 15% higher growth rate of hoof horn and 15% more hoof growth, at the midline dead centre, by the end of the trial period.

Differences in total growth of hoof horn between feet and between ponies

Analysis of variance (ANOVA) was carried out on total growth, by foot, and by individual pony, for treatment and control animals.

This showed that there was no difference between feet for all 8 ponies but that there was a difference between individuals for total growth ($P<0.05$). The 2 older animals (*pony 4* and *pony 8* which comprised pair 4) both had significantly reduced hoof growth compared to the rest by the end of the trial.

Discussion

Experimental design

Restriction of experimental error in feeding trial design is desirable and is aided by controlling the extent of variability between animals. Experimental variabilty can be reduced by matching animals as nearly as possible (Roberts 1975). This has long been a principle in agricultural experiments. For example with dairy cows, matching by date of calving, parity, size, and previous milk yield are important and has been done for experiments leading to foot measurements in cattle (Reilly and Brooks 1990). For this experiment, because of the species used, and the nature of the measurements, it was more appropriate to match for breed, age, sex, size and weight. These are similar to the important factors to match for in beef trials (Roberts 1975). 'Growth' of horn is therefore analogous to 'weight' of beef animal in such trials, and 'growth rate' of horn is analogous to 'growth rate' of the beast.

In order to control for the vagaries of horse feeding in terms of variable inputs from hard feed, pasture or hay for example, a commercial cube was chosen as the basal diet and as the carrier for biotin supplementation. Although the plasma kinetics for feeding biotin in other forms are known, this is not so for this experiment, as was the case with Buffa *et al.* (1992), because of the prohibitively high cost of plasma biotin analysis. Initial hoof growth measurement between the potential treatment and control groups was carried out in only the last 26 day period of basal feeding as assessment prior to then may have recorded the effects of nutritional and environmental factors affecting horn growth prior to purchase. The fact that there was no difference between the 2 groups meant that they could be confidently allocated without any carry over of covariate confounding factors. This acted as a form of internal control.

Hoof horn growth

Both CB and RH have been used as reference points in the assessment of hoof horn growth which is subject to potential measurement error. Geyer and Schulze (1994) used the CB and Butler and Hintz (1977), Graham *et al.* (1994) and Josseck *et al.*

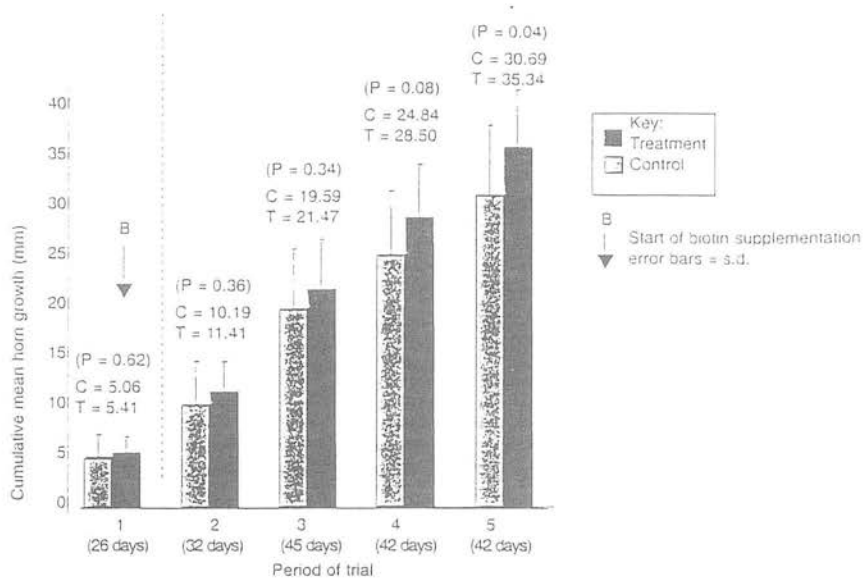


Fig 2: Cumulative mean hoof growth (all feet) by period of trial.

(1995) all used RH. The dividers and steel ruler method was used to improve the accuracy of measurement in this work, but the repeatability of measurements also needed to be addressed. A pilot study established that the repeatability of measurements to RH was within 1% whereas for the CB it was >4%. The latter method was therefore rejected.

The effect of biotin on hoof horn growth in this trial is in agreement with the findings of Bains (1985) and Buffa *et al.* (1992). Can biotin be considered, then, a therapy? It can in so far as it has produced a change that will alter the 'renewal time' (Geyer and Schulze 1994) of the capsule for these ponies. From the probability of the differences in growth between control and treatment groups over time (Fig 2) it is possible that the effect on the renewal time of the capsule would be more marked should supplementation have continued for longer. In addition, a small but significant difference in mean growth of 4.65 mm in a treated foot compared to a nontreated foot over a period of 5 months, may just be sufficient to make a difference in new horn for nailing in to in an overwintered animal, or to push away an old lesion which, as a result of the rather subtle mechanism of hoof wall growth and repair (Reilly *et al.* 1996), does not rely on total capsular renewal to be replaced. In situations where total capsule regrowth is required, then biotin supplementation may help reduce 'renewal times' following hoof capsule avulsions or perhaps after major resections subsequent to laminitis or onychomycosis. This, however, assumes a similar response in resected horn which cannot be inferred from the results of this work. Further work is required to investigate the effects of biotin as a therapy under these circumstances.

Hoof growth differences between feet and between individuals

There was no difference in growth between feet in this trial. This does not agree with Butler (1976), and Scott and Butler (1980). However, these authors reported growth in foal groups as they aged from 8 months. The ponies in this trial were more mature. There is no clear evidence as to whether or not growth decreases with older age within the equid population, other than foals and yearlings appearing to have higher growth rates when a comparison between different workers' results is made from Table 1.

In this trial, there was a difference between individuals by ANOVA, with the 2 older ponies showing a significantly decreased growth of horn compared to the others. Further work is required from trials designed to investigate the relationship between age of animal and response to biotin supplementation.

Hoof horn growth rates

The mean growth rates in this trial of 0.164 mm/day (control) and 0.189 mm/day (treated) over a winter period (October–February) are in agreement with a generalised figure, for mature horses, of 0.2 mm/day (Table 1) and with Glade and Saltzmann (1985). This is lower than those recorded by Buffa *et al.* (1992) but that investigation was conducted in South Africa, in a dry season and with horses that were exercised and shod.

Pollitt (1990) showed an inferred difference in hoof growth rate by dorsopalmar depth into the dorsal hoof wall, with an autoradiographic technique. Measuring the rate of descent of a branded mark on the outside of the hoof capsule may only indicate part of what is happening within the hoof wall. Evidence for 'zonation' by dorsopalmar depth of the wall has been given by Reilly *et al.* (1996).

How biotin exerts its effect is unclear. Buffa *et al.* (1992) concluded that it influenced the amount or proportion of keratin molecules in hoof horn, resulting in increased growth rate. Kempson (1989), citing Marston (1946), implied that a response to biotin supplementation was brought about 'through modification of the division and maturation of proliferative cells'. To what extent biotin has effects during the cornification

and/or keratinisation processes cannot be concluded until measurement is undertaken at the cellular/molecular level. Otherwise, it can be postulated only that alleviation of a rate limiting step in the enzymatic functions of biotin may explain the responses in this trial.

This was a biotin super supplementation experiment to a group of ponies that did not have overtly problem affected feet. The lack of rationale for a response to super supplementation in horses that should not theoretically be biotin deficient has been discussed by Buffa *et al.* (1992). In that report the horses had 'poor hoof horn' by visual inspection, in this case the ponies did not. They were also not being worked, nor unduly stressed, and a response to supplementation has, therefore, been shown in a group of animals that should not have been biotin deficient and for which confounding factors had been attempted to be minimised. Control ponies were receiving approximately 0.3 mg of biotin/day (Table 2) and the Treatment animals were receiving approximately 30 mg of biotin/day. This is equivalent to approximately 60 mg/day if extrapolated to a 500 kg horse. The 'requirement' for biotin is not known but the control animals were receiving an acceptable level of biotin for maintainance according to Harris *et al.* (1995) who recommend 0.1 mg/kg of diet. Therefore, the results from this trial can be regarded as a supraoptimal response (Cuddeford 1991) in ponies with normal feet. It is unknown whether a higher dose rate of biotin would effect bigger differences. Further work is required to be able to assess fully the response of the equid to supraoptimal supplementation with keratogenous factors. Future approaches to experimental work should, ideally, include double blinding and cross over design.

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APPENDIX III

APPENDIX III: Macro programme for semi-automization of computerized image anaylsis

```
macro 'Copy Grey Image [C]';
begin;
SelectAll;
SaveAs('BobsHardDisk: NIH-Image(PPC):Macros:TempGrey');
end;

macro 'Measure [M]';
begin;
MakeBinary;
Erode;
Dilate;
SetOptions('Area, Major, Minor');
LabelParticles(true);
OutlineParticles(true);
IgnoreParticlesTouchingEdge(false);
IncludeInteriorHoles(true);
SetParticleSize(100,999999);
AnalyzeParticles;
end;

macro 'Add Grey Image [A]';
begin;
Open ('BobsHardDisk: NIH-Image(PPC):Macros:TempGrey');
SelectAll;
Copy;
Close;
Paste;
DoXor;
end;
```

Author: Mr Bob Fleming
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APPENDIX IV

KEY FOR PEARSON CORRELATION COEFFICIENT MATRIX

(the use of these terms in the matrix are additive)

WT *075	Pony bodyweight to the power of 0.75 (ignore)
Weight	Pony bodyweight
MA-Z1, Z2, Z3, Z4	Marrow absolute size in Zones1, 2, 3 or 4
TU-Z1, Z2, Z3, Z4	Tubule absolute size in Zones1, 2, 3 or 4
CO-Z1, Z2, Z3, Z4	Cortex absolute size in Zones1, 2, 3 or 4
MTU-Z1, Z2, Z3, Z4	Median tubule size in Zones1, 2, 3 or 4
MCO-Z1, Z2, Z3, Z4	Median cortex size in Zones1, 2, 3 or 4
CFC1	Moisture content in fresh beam cut 1
RFC1	Moisture regain in fresh beam cut 1
CHC1	Moisture content in hydrated beam cut 1
RHC1	Moisture regain in hydrated beam cut 1
GP1,2,3,4,5	Hoof horn growth in periods 1, 2, 3, 4 or 5.
GRP1,2,3,4,5	Hoof horn growth rate in periods 1, 2, 3, 4 or 5.
TG	Total growth for trial
TGR	Mean growth rate for trial
Z1, Z2, Z3, Z4 MEAN	Mean tubule density in Zones 1, 2, 3 or 4
ZAF	Zonal area fraction
IT	Intertubular horn
Fb 1	Stiffness value from fresh bend 1
HB 1	Stiffness value from fully hydrated bend 1
Db 1	Stiffness value from dry bend 1

Correlations (Pearson)

	AGE	WT*075	Weight	MA-Z1	TU-Z1	CO-Z1	MTU-Z1	MCO-Z1
WT*075	-0.915 0.001							
Weight	-0.913 0.002	1.000 0.000						
MA-Z1	-0.305 0.462	0.473 0.237	0.474 0.236					
TU-Z1	-0.485 0.223	0.680 0.063	0.690 0.058	0.387 0.344				
CO-Z1	-0.460 0.252	0.633 0.092	0.643 0.086	0.223 0.596	0.985 0.000			
MTU-Z1	-0.521 0.185	0.739 0.036	0.747 0.033	0.507 0.200	0.987 0.000	0.949 0.000		
MCO-Z1	-0.501 0.206	0.701 0.053	0.710 0.049	0.328 0.428	0.994 0.000	0.989 0.000	0.980 0.000	
MA-Z2	-0.320 0.440	0.456 0.257	0.453 0.260	0.906 0.002	0.238 0.570	0.083 0.845	0.347 0.400	0.174 0.681
TU-Z2	-0.855 0.007	0.883 0.004	0.888 0.003	0.551 0.157	0.680 0.064	0.619 0.102	0.726 0.041	0.663 0.073
CO-Z2	-0.863 0.006	0.872 0.005	0.878 0.004	0.447 0.266	0.690 0.058	0.649 0.082	0.723 0.043	0.683 0.062
MTU-Z2	-0.857 0.007	0.840 0.009	0.846 0.008	0.546 0.161	0.667 0.071	0.607 0.111	0.700 0.053	0.640 0.087
MCO-Z2	-0.851 0.007	0.818 0.013	0.825 0.012	0.438 0.277	0.675 0.066	0.635 0.091	0.699 0.054	0.663 0.073
MA-Z3	-0.450 0.263	0.633 0.092	0.633 0.092	0.500 0.207	0.547 0.161	0.485 0.223	0.589 0.124	0.522 0.185
TU-Z3	-0.686 0.061	0.744 0.034	0.743 0.035	0.700 0.053	0.599 0.117	0.504 0.202	0.654 0.078	0.552 0.156
CO-Z3	-0.504 0.203	0.351 0.393	0.346 0.402	0.478 0.231	-0.077 0.856	-0.168 0.691	0.002 0.994	-0.116 0.784
MTU-Z3	-0.537 0.170	0.692 0.057	0.693 0.057	0.792 0.019	0.539 0.168	0.423 0.296	0.624 0.098	0.495 0.212
MCO-Z3	-0.470 0.240	0.683 0.062	0.684 0.062	0.850 0.007	0.560 0.149	0.434 0.283	0.658 0.076	0.521 0.185
MA-Z4	-0.784 0.027	0.841 0.009	0.843 0.008	0.205 0.626	0.738 0.037	0.743 0.035	0.747 0.033	0.761 0.028
TU-Z4	-0.643 0.272	0.513 0.193	0.525 0.182	0.301 0.469	0.899 0.002	0.896 0.003	0.846 0.008	0.859 0.006
CO-Z4	-0.417 0.304	0.486 0.222	0.498 0.210	0.290 0.486	0.887 0.003	0.885 0.004	0.830 0.011	0.844 0.008

MTU-Z4	-0.484	0.506	0.518	0.062	0.858	0.897	0.783	0.843
	0.224	0.201	0.189	0.885	0.006	0.003	0.022	0.009
MCO-Z4	-0.049	0.048	0.042	-0.483	-0.223	-0.146	-0.268	-0.185
	0.908	0.910	0.922	0.226	0.595	0.729	0.520	0.661
CFC1	0.352	-0.239	-0.224	0.136	0.253	0.241	0.205	0.188
	0.393	0.569	0.594	0.748	0.546	0.565	0.626	0.656
RFC1	0.395	-0.285	-0.270	0.111	0.229	0.221	0.179	0.166
	0.333	0.494	0.518	0.793	0.585	0.599	0.671	0.695

CHC1	0.418	-0.543	-0.540	-0.032	-0.396	-0.412	-0.434	-0.478
	0.303	0.164	0.167	0.941	0.331	0.310	0.283	0.231
RHC1	0.428	-0.554	-0.551	-0.036	-0.399	-0.415	-0.438	-0.481
	0.291	0.155	0.157	0.933	0.327	0.307	0.278	0.228

GP1	-0.193	0.144	0.132	-0.287	-0.399	-0.368	-0.369	-0.329
	0.646	0.734	0.755	0.491	0.328	0.370	0.368	0.426
GRP1	-0.194	0.145	0.133	-0.288	-0.397	-0.366	-0.368	-0.327
	0.645	0.732	0.753	0.489	0.330	0.373	0.370	0.429
GP2	-0.183	0.113	0.105	-0.443	-0.160	-0.087	-0.166	-0.069
	0.664	0.789	0.804	0.271	0.705	0.838	0.695	0.872
GRP2	-0.183	0.113	0.105	-0.441	-0.161	-0.088	-0.166	-0.070
	0.665	0.790	0.804	0.274	0.703	0.835	0.694	0.870
GP3	-0.486	0.647	0.650	0.106	0.879	0.909	0.849	0.906
	0.222	0.083	0.081	0.804	0.004	0.002	0.008	0.002
GRP3	-0.490	0.649	0.652	0.105	0.879	0.909	0.849	0.906
	0.218	0.082	0.080	0.805	0.004	0.002	0.008	0.002
GP4	-0.321	0.419	0.415	0.296	0.145	0.098	0.211	0.146

	0.438	0.302	0.307	0.476	0.732	0.817	0.616	0.731
GRP4	-0.322	0.420	0.416	0.296	0.145	0.098	0.211	0.146
	0.437	0.300	0.306	0.476	0.731	0.817	0.615	0.730
GP5	0.428	-0.652	-0.654	-0.865	-0.587	-0.459	-0.691	-0.553
	0.290	0.079	0.079	0.006	0.126	0.252	0.058	0.155
GRP5	0.426	-0.651	-0.653	-0.864	-0.584	-0.456	-0.688	-0.550
	0.293	0.080	0.079	0.006	0.129	0.256	0.059	0.158
TG	-0.397	0.387	0.377	-0.442	0.030	0.113	0.020	0.126
	0.330	0.344	0.357	0.273	0.945	0.789	0.962	0.766
TGR	-0.395	0.385	0.375	-0.446	0.030	0.114	0.020	0.126
	0.332	0.347	0.360	0.268	0.944	0.787	0.963	0.766
Z1MEAN	0.185	-0.507	-0.517	-0.374	-0.904	-0.884	-0.908	-0.911
	0.660	0.199	0.190	0.362	0.002	0.004	0.002	0.002
Z2MEAN	0.684	-0.833	-0.839	-0.747	-0.780	-0.686	-0.844	-0.749
	0.061	0.010	0.009	0.033	0.023	0.060	0.008	0.032
Z3MEAN	0.787	-0.752	-0.750	-0.738	-0.450	-0.341	-0.520	-0.402
	0.020	0.031	0.032	0.037	0.264	0.409	0.187	0.324
Z4MEAN	0.440	-0.413	-0.411	-0.350	-0.272	-0.224	-0.316	-0.284
	0.275	0.309	0.312	0.395	0.515	0.594	0.446	0.495
ZAFMAZ1	-0.328	0.239	0.233	0.737	-0.164	-0.309	-0.054	-0.229
	0.428	0.569	0.578	0.037	0.697	0.457	0.900	0.585
ZAFMAZ2	0.493	-0.417	-0.428	-0.041	-0.556	-0.582	-0.531	-0.570
	0.214	0.304	0.290	0.923	0.153	0.130	0.176	0.140
ZAFMAZ3	0.017	0.143	0.143	-0.148	0.374	0.421	0.350	0.404
	0.968	0.735	0.736	0.727	0.361	0.299	0.395	0.321
ZAFMAZ4	-0.469	0.421	0.412	0.073	-0.132	-0.151	-0.072	-0.111
	0.242	0.299	0.311	0.864	0.756	0.721	0.865	0.794
ZAFTUZ1	-0.653	0.674	0.677	0.200	0.841	0.853	0.804	0.835
	0.079	0.067	0.065	0.634	0.009	0.007	0.016	0.010
ZAFTUZ2	-0.421	0.449	0.443	-0.029	0.070	0.080	0.101	0.101
	0.299	0.264	0.272	0.946	0.868	0.850	0.812	0.812
ZAFTUZ3	-0.232	0.231	0.236	0.199	0.537	0.531	0.510	0.501
	0.580	0.582	0.573	0.637	0.170	0.176	0.197	0.206
ZAFTUZ4	-0.343	0.308	0.314	0.456	0.409	0.350	0.394	0.421
	0.406	0.457	0.449	0.256	0.314	0.396	0.334	0.438
ZAFCOZ1	-0.515	0.564	0.568	-0.032	0.851	0.906	0.780	0.863
	0.191	0.146	0.141	0.940	0.007	0.002	0.022	0.006
ZAFCOZ2	-0.661	0.651	0.650	-0.120	0.279	0.319	0.283	0.324
	0.075	0.080	0.081	0.778	0.503	0.441	0.497	0.434
ZAFCOZ3	-0.265	0.220	0.225	0.218	0.510	0.500	0.486	0.473
	0.526	0.600	0.593	0.604	0.196	0.207	0.222	0.236
ZAFCOZ4	-0.306	0.273	0.280	0.459	0.429	0.371	0.409	0.338
	0.460	0.512	0.502	0.253	0.288	0.366	0.315	0.413
ZAFITZ.	0.653	-0.674	-0.677	-0.201	-0.841	-0.853	-0.804	-0.835
	0.079	0.067	0.065	0.634	0.009	0.007	0.016	0.010

ZAFITZ2	0.469	-0.490	-0.484	0.138	-0.061	-0.091	-0.075	-0.101
	0.241	0.218	0.224	0.744	0.886	0.830	0.861	0.812
ZAFITZ3	0.237	-0.228	-0.235	-0.176	-0.603	-0.605	-0.560	-0.563
	0.572	0.587	0.575	0.676	0.114	0.112	0.149	0.146
ZAFITZ4	0.347	-0.318	-0.323	-0.468	-0.402	-0.340	-0.395	-0.317
	0.399	0.442	0.435	0.242	0.324	0.410	0.333	0.444
Fb 1	-0.921	0.731	0.729	0.238	0.313	0.290	0.342	0.317
	0.001	0.039	0.040	0.570	0.451	0.486	0.407	0.444
Hb 1	-0.755	0.611	0.615	-0.156	0.388	0.442	0.356	0.416
	0.030	0.108	0.104	0.712	0.343	0.272	0.387	0.305
Db 1	0.203	-0.019	-0.010	-0.041	0.013	0.020	0.050	0.060
	0.629	0.964	0.982	0.923	0.976	0.963	0.906	0.888

	MA-Z2	TU-Z2	CO-Z2	MTU-Z2	MCO-Z2	MA-Z3	TU-Z3	CO-Z3
TU-Z2	0.497							
	0.211							
CO-Z2	0.375	0.991						
	0.360	0.000						
MTU-Z2	0.477	0.971	0.962					
	0.232	0.000	0.000					
MCO-Z2	0.310	0.949	0.965	0.979				
	0.455	0.000	0.000	0.000				
MA-Z3	0.669	0.656	0.598	0.527	0.466			
	0.070	0.077	0.117	0.179	0.315			
TU-Z3	0.762	0.828	0.766	0.777	0.668	0.842		
	0.028	0.011	0.027	0.023	0.070	0.009		
CO-Z3	0.529	0.545	0.499	0.486	0.422	0.398	0.663	
	0.178	0.162	0.208	0.222	0.297	0.329	0.073	
MTU-Z3	0.855	0.779	0.700	0.689	0.567	0.906	0.939	0.605
	0.007	0.023	0.053	0.059	0.143	0.002	0.001	0.112
MCO-Z3	0.882	0.720	0.633	0.630	0.508	0.874	0.886	0.501
	0.004	0.044	0.092	0.094	0.199	0.005	0.003	0.206
MA-Z4	0.191	0.848	0.876	0.739	0.753	0.717	0.706	0.344
	0.650	0.008	0.004	0.036	0.031	0.045	0.050	0.404

TU-Z4	0.196	0.664	0.679	0.718	0.713	0.453	0.582	-0.011
	0.641	0.072	0.064	0.045	0.047	0.260	0.130	0.980
CO-Z4	0.192	0.637	0.652	0.698	0.690	0.437	0.562	-0.038
	0.649	0.089	0.080	0.054	0.058	0.279	0.147	0.929
MTU-Z4	-0.055	0.638	0.690	0.683	0.726	0.336	0.441	-0.079
	0.897	0.089	0.058	0.062	0.041	0.415	0.274	0.853
MCO-Z4	-0.119	-0.153	-0.145	-0.205	-0.263	0.258	-0.126	-0.261
	0.778	0.717	0.732	0.626	0.529	0.537	0.766	0.532
CFC1	0.091	0.091	0.083	0.133	0.111	0.145	-0.001	-0.247
	0.830	0.830	0.844	0.753	0.794	0.732	0.997	0.555
RFC1	0.051	0.044	0.039	0.091	0.076	0.087	-0.053	-0.280
	0.905	0.918	0.927	0.830	0.858	0.837	0.900	0.502

GP1	-0.115	-0.245	-0.244	-0.230	-0.235	-0.217	-0.309	-0.212
	0.786	0.559	0.561	0.584	0.575	0.605	0.457	0.614
GRP1	-0.116	-0.244	-0.243	-0.229	-0.235	-0.216	-0.308	-0.213
	0.785	0.561	0.562	0.585	0.576	0.608	0.458	0.612
GP2	-0.497	-0.272	-0.214	-0.241	-0.141	-0.498	-0.442	-0.353
	0.210	0.514	0.611	0.565	0.740	0.210	0.273	0.391
GRP2	-0.495	-0.273	-0.215	-0.241	-0.141	-0.498	-0.442	-0.354
	0.213	0.513	0.609	0.565	0.739	0.209	0.273	0.390

GP3	0.035	0.509	0.539	0.443	0.465	0.533	0.516	-0.094
	0.935	0.198	0.168	0.272	0.246	0.173	0.191	0.825
GRP3	0.034	0.510	0.541	0.445	0.467	0.533	0.517	-0.093
	0.935	0.196	0.166	0.269	0.243	0.174	0.190	0.827
GP4	0.445	0.485	0.450	0.281	0.210	0.803	0.626	0.631
	0.270	0.223	0.263	0.500	0.617	0.016	0.097	0.093
GRP4	0.445	0.486	0.451	0.282	0.211	0.804	0.626	0.631
	0.269	0.222	0.263	0.498	0.616	0.016	0.097	0.094
GP5	-0.815	-0.712	-0.635	-0.608	-0.516	-0.804	-0.850	-0.518
	0.014	0.047	0.091	0.109	0.190	0.016	0.008	0.189
GRP5	-0.815	-0.709	-0.632	-0.605	-0.513	-0.804	-0.847	-0.517
	0.014	0.049	0.093	0.112	0.194	0.016	0.008	0.190
TG	-0.332	-0.004	0.048	-0.080	-0.028	0.047	-0.083	-0.163
	0.422	0.993	0.910	0.851	0.947	0.913	0.846	0.699
TGR	-0.336	-0.004	0.048	-0.082	-0.030	0.049	-0.083	-0.163
	0.416	0.992	0.910	0.848	0.944	0.909	0.845	0.699
Z1MEAN	-0.210	-0.430	-0.428	-0.388	-0.395	-0.476	-0.367	0.345
	0.617	0.288	0.290	0.343	0.332	0.233	0.371	0.403
Z2MEAN	-0.666	-0.934	-0.895	-0.894	-0.841	-0.747	-0.869	-0.439
	0.071	0.001	0.003	0.003	0.009	0.033	0.005	0.277
Z3MEAN	-0.771	-0.820	-0.756	-0.840	-0.739	-0.609	-0.918	-0.717
	0.025	0.013	0.030	0.009	0.036	0.109	0.001	0.045
Z4MEAN	-0.155	-0.209	-0.199	-0.346	-0.374	0.279	-0.107	0.048
	0.714	0.619	0.637	0.401	0.362	0.503	0.800	0.911
ZAFMAZ1	0.782	0.394	0.298	0.407	0.291	0.285	0.585	0.843
	0.022	0.334	0.473	0.317	0.485	0.493	0.127	0.009
ZAFMAZ2	0.269	-0.571	-0.651	-0.622	-0.760	0.126	-0.162	-0.100
	0.519	0.140	0.080	0.100	0.028	0.766	0.701	0.813
ZAFMAZ3	-0.118	0.115	0.143	-0.081	-0.065	0.528	0.268	0.051
	0.781	0.787	0.736	0.849	0.879	0.179	0.521	0.905
ZAFMAZ4	0.306	0.402	0.382	0.237	0.176	0.602	0.485	0.680
	0.462	0.323	0.350	0.573	0.677	0.114	0.224	0.063
ZAFTUZ1	0.146	0.669	0.693	0.655	0.657	0.531	0.699	0.207
	0.733	0.070	0.057	0.078	0.076	0.176	0.054	0.623
ZAFTUZ2	0.197	0.410	0.408	0.218	0.172	0.705	0.478	0.486
	0.647	0.313	0.316	0.605	0.684	0.049	0.231	0.223
ZAFTUZ3	0.106	0.479	0.496	0.397	0.406	0.472	0.594	0.461
	0.803	0.230	0.211	0.330	0.318	0.237	0.121	0.250
ZAFTUZ4	0.571	0.622	0.575	0.642	0.529	0.653	0.752	0.498
	0.140	0.100	0.136	0.086	0.177	0.079	0.031	0.209
ZAFCOZ1	-0.093	0.516	0.567	0.508	0.543	0.411	0.476	-0.085
	0.826	0.191	0.143	0.199	0.164	0.312	0.232	0.842
ZAFCOZ2	0.033	0.622	0.659	0.487	0.495	0.609	0.441	0.343
	0.939	0.100	0.075	0.221	0.212	0.109	0.274	0.406
ZAFCOZ3	0.082	0.467	0.486	0.417	0.438	0.347	0.568	0.492

	0.846	0.244	0.222	0.304	0.278	0.399	0.142	0.216
ZAFC024	0.552	0.593	0.548	0.633	0.524	0.601	0.720	0.443
	0.156	0.121	0.160	0.092	0.183	0.115	0.044	0.272
ZAFITZ1	-0.146	-0.669	-0.693	-0.656	-0.658	-0.531	-0.699	-0.208
	0.730	0.070	0.057	0.078	0.076	0.176	0.054	0.622
ZAFITZ2	-0.135	-0.398	-0.404	-0.243	-0.197	-0.657	-0.376	-0.303
	0.749	0.329	0.321	0.563	0.640	0.077	0.359	0.466
ZAFITZ3	-0.064	-0.491	-0.515	-0.442	-0.458	-0.424	-0.568	-0.374
	0.880	0.216	0.191	0.273	0.254	0.295	0.142	0.362
ZAFITZ4	-0.582	-0.632	-0.585	-0.628	-0.516	-0.694	-0.788	-0.566
	0.130	0.092	0.128	0.095	0.191	0.056	0.020	0.143
Fb 1	0.215	0.784	0.803	0.788	0.803	0.298	0.638	0.706
	0.609	0.021	0.016	0.020	0.016	0.473	0.089	0.051
Hb 1	-0.179	0.723	0.800	0.698	0.776	0.263	0.358	0.340
	0.671	0.043	0.017	0.054	0.023	0.530	0.383	0.410
Db 1	-0.231	-0.020	0.015	-0.058	0.040	-0.219	-0.440	-0.418
	0.581	0.962	0.973	0.891	0.924	0.602	0.275	0.303

	MTU-Z3	MCO-Z3	MA-Z4	TU-Z4	CO-Z4	MTU-Z4	MCO-Z4	CFC1
MCO-Z3	0.982							
	0.000							
MA-Z4	0.645	0.582						
	0.084	0.130						
TU-Z4	0.460	0.424	0.618					
	0.251	0.295	0.103					
CO-Z4	0.440	0.405	0.586	0.989				
	0.275	0.319	0.127	0.000				
MTU-Z4	0.291	0.235	0.677	0.936	0.951			
	0.485	0.575	0.065	0.000	0.000			
MCO-Z4	-0.084	-0.120	0.080	-0.233	-0.222	-0.148		
	0.842	0.778	0.851	0.578	0.597	0.726		
CFC1	0.126	0.119	-0.056	0.487	0.488	0.390	-0.108	
	0.766	0.779	0.895	0.213	0.220	0.340	0.800	
RFC1	0.073	0.071	-0.101	0.444	0.465	0.370	-0.137	0.998

	0.863	0.867	0.812	0.271	0.246	0.366	0.747	0.000
CHC1	0.055	-0.046	-0.318	-0.059	-0.041	-0.140	0.044	0.583
	0.898	0.915	0.442	0.889	0.923	0.741	0.917	0.129
RHC1	0.044	-0.055	-0.329	-0.061	-0.042	-0.141	0.037	0.583
	0.917	0.897	0.426	0.887	0.921	0.738	0.930	0.129
GP1	-0.305	-0.259	-0.182	-0.559	-0.557	-0.474	0.599	-0.656
	0.462	0.535	0.666	0.149	0.152	0.235	0.117	0.077
GRP1	-0.305	-0.259	-0.180	-0.558	-0.555	-0.472	0.601	-0.655
	0.463	0.536	0.669	0.151	0.154	0.238	0.115	0.078
GP2	-0.547	-0.491	-0.092	-0.364	-0.370	-0.200	0.191	-0.755
	0.161	0.216	0.828	0.375	0.367	0.636	0.651	0.030
GRP2	-0.547	-0.491	-0.094	-0.365	-0.370	-0.201	0.191	-0.755
	0.161	0.217	0.824	0.374	0.366	0.633	0.651	0.030
GP3	0.403	0.409	0.783	0.698	0.680	0.723	-0.010	-0.108
	0.323	0.315	0.022	0.054	0.064	0.043	0.981	0.800
GRP3	0.403	0.408	0.784	0.699	0.681	0.725	-0.008	-0.109
	0.323	0.316	0.021	0.054	0.063	0.042	0.985	0.798
GP4	0.726	0.664	0.621	0.014	-0.017	-0.021	0.206	-0.087
	0.041	0.073	0.100	0.974	0.969	0.961	0.625	0.838
GRP4	0.727	0.665	0.622	0.014	-0.016	-0.020	0.208	-0.086
	0.041	0.072	0.100	0.973	0.970	0.962	0.622	0.840
GP5	-0.949	-0.976	-0.591	-0.420	-0.396	-0.242	0.290	-0.105
	0.000	0.000	0.123	0.300	0.332	0.563	0.486	0.805
GRP5	-0.948	-0.976	-0.589	-0.414	-0.390	-0.237	0.288	-0.102
	0.000	0.000	0.124	0.308	0.340	0.572	0.488	0.810

GRP1	-0.658	-0.211	-0.256	0.479	0.511	-0.500	-0.502	-0.224
	0.076	0.617	0.541	0.230	0.195	0.207	0.205	0.594
GP2	-0.732	0.027	-0.016	0.008	0.020	-0.784	-0.781	-0.196
	0.039	0.949	0.970	0.986	0.962	0.021	0.022	0.642
GRP2	-0.732	0.026	-0.017	0.010	0.022	-0.784	-0.781	-0.195
	0.039	0.951	0.968	0.982	0.958	0.021	0.022	0.643
GP3	-0.130	-0.330	-0.332	-0.736	-0.732	-0.542	-0.544	-0.710
	0.759	0.425	0.421	0.037	0.039	0.166	0.163	0.049
GRP3	-0.131	-0.329	-0.331	-0.737	-0.732	-0.542	-0.544	-0.710
	0.757	0.427	0.423	0.037	0.039	0.165	0.163	0.049
GP4	-0.133	-0.510	-0.495	0.184	0.195	0.167	0.155	-0.510
	0.753	0.197	0.213	0.662	0.643	0.692	0.714	0.197
GRP4	-0.132	-0.510	-0.495	0.185	0.196	0.167	0.155	-0.510
	0.755	0.196	0.213	0.661	0.641	0.692	0.714	0.197
GP5	-0.065	0.724	0.679	-0.009	0.001	0.094	0.102	0.646
	0.878	0.042	0.064	0.983	0.999	0.825	0.810	0.084
GRP5	-0.063	0.727	0.681	-0.014	-0.004	0.096	0.104	0.647
	0.883	0.041	0.063	0.974	0.992	0.821	0.806	0.083
TG	-0.759	-0.223	-0.271	-0.050	-0.019	-0.697	-0.701	-0.536
	0.029	0.596	0.516	0.906	0.964	0.055	0.053	0.171
TGR	-0.757	-0.219	-0.268	-0.054	-0.023	-0.693	-0.697	-0.534
	0.030	0.602	0.521	0.900	0.957	0.057	0.055	0.173
Z1MEAN	-0.311	0.331	0.272	0.599	0.606	0.455	0.457	0.496
	0.454	0.423	0.515	0.117	0.111	0.257	0.255	0.211
Z2MEAN	-0.174	0.448	0.415	0.278	0.262	0.168	0.177	0.524
	0.680	0.265	0.306	0.505	0.530	0.692	0.675	0.182
Z3MEAN	0.171	0.515	0.540	-0.007	-0.022	0.059	0.067	0.523
	0.685	0.191	0.167	0.987	0.959	0.889	0.875	0.183
Z4MEAN	0.402	0.199	0.221	0.092	0.096	0.683	0.678	0.270
	0.324	0.637	0.598	0.828	0.821	0.062	0.065	0.518
ZAFMAZ1	-0.149	-0.431	-0.442	0.552	0.544	0.299	0.295	-0.144
	0.725	0.286	0.273	0.156	0.163	0.471	0.478	0.734
ZAFMAZ2	-0.120	-0.428	-0.441	0.694	0.678	0.319	0.319	-0.103
	0.778	0.290	0.274	0.056	0.065	0.442	0.442	0.808
ZAFMAZ3	-0.075	-0.259	-0.244	-0.388	-0.402	-0.072	-0.076	-0.485
	0.859	0.536	0.561	0.342	0.324	0.865	0.859	0.224
ZAFMAZ4	-0.432	-0.406	-0.431	0.365	0.396	0.093	0.081	-0.442
	0.285	0.318	0.286	0.374	0.331	0.826	0.849	0.273
ZAFTUZ1	-0.109	-0.223	-0.250	-0.742	-0.733	-0.322	-0.324	-0.592
	0.797	0.596	0.550	0.035	0.039	0.437	0.434	0.122
ZAFTUZ2	-0.313	-0.386	-0.400	0.129	0.161	0.033	0.020	-0.515
	0.450	0.345	0.326	0.761	0.704	0.938	0.963	0.192
ZAFTUZ3	0.160	-0.037	-0.034	-0.578	-0.596	0.208	0.207	-0.239
	0.705	0.931	0.936	0.133	0.119	0.621	0.623	0.569
ZAFTUZ4	0.484	-0.093	-0.099	-0.213	-0.199	0.567	0.561	-0.061

	0.224	0.826	0.816	0.613	0.637	0.143	0.148	0.886
ZAFCOZ1	-0.023	-0.062	-0.085	-0.872	-0.860	-0.384	-0.385	-0.493
	0.956	0.883	0.842	0.005	0.006	0.348	0.347	0.215
ZAFCOZ2	-0.214	-0.131	-0.144	-0.141	-0.085	-0.145	-0.159	-0.405
	0.610	0.758	0.733	0.739	0.841	0.732	0.707	0.319
ZAFCOZ3	0.091	0.006	0.000	-0.585	-0.606	0.158	0.158	-0.201
	0.831	0.989	1.000	0.128	0.111	0.709	0.708	0.633
ZAFCOZ4	0.529	-0.055	-0.059	-0.254	-0.243	0.565	0.560	-0.020
	0.177	0.897	0.889	0.544	0.562	0.145	0.149	0.963
ZAFITZ1	0.109	0.223	0.250	0.741	0.733	0.322	0.324	0.592
	0.797	0.596	0.550	0.035	0.039	0.437	0.434	0.122
ZAFITZ2	0.267	0.299	0.318	-0.131	-0.181	0.025	0.039	0.449
	0.523	0.472	0.442	0.757	0.668	0.953	0.927	-0.265
ZAFITZ3	-0.249	-0.053	-0.055	0.680	0.696	-0.205	-0.205	0.173
	0.553	0.900	0.896	0.063	0.055	0.627	0.627	0.682
ZAFITZ4	-0.431	0.142	0.147	0.196	0.185	-0.564	-0.558	0.114
	0.287	0.737	0.729	0.641	0.660	0.145	0.151	0.787
Fb 1	-0.423	-0.096	-0.145	-0.186	-0.151	-0.229	-0.236	-0.345
	0.296	0.820	0.732	0.660	0.722	0.585	0.573	0.403
Hb 1	-0.063	0.299	0.278	-0.463	-0.407	-0.141	-0.150	-0.071
	0.883	0.471	0.506	0.248	0.316	0.740	0.724	0.866
Db 1	0.355	0.255	0.343	0.049	0.063	-0.226	-0.229	0.308
	0.388	0.543	0.406	0.908	0.883	0.591	0.585	0.457
Fb 2	-0.355	0.045	-0.010	-0.287	-0.234	-0.292	-0.300	-0.274
	0.389	0.916	0.982	0.491	0.577	0.483	0.471	0.512
Hb 2	-0.165	0.167	0.140	-0.310	-0.247	-0.213	-0.224	-0.162
	0.696	0.692	0.741	0.455	0.555	0.613	0.595	0.702
Db 2	-0.093	-0.053	-0.005	0.582	0.556	-0.230	-0.225	0.253
	0.827	0.900	0.990	0.130	0.152	0.584	0.592	0.545
Fb 3	-0.334	0.014	-0.032	-0.344	-0.297	-0.306	-0.308	-0.317
	0.419	0.974	0.940	0.405	0.476	0.476	0.458	0.443
H b3	-0.196	0.154	0.125	-0.258	-0.191	-0.220	-0.231	-0.175
	0.641	0.715	0.768	0.537	0.650	0.601	0.582	0.679
D b3	0.011	-0.072	-0.001	0.493	0.496	-0.305	-0.308	0.144
	0.979	0.866	0.999	0.215	0.211	0.462	0.457	0.734

	RHC2	CHC3	RHC3	TYPE	SEX	GP1	GRP1	GP2
CHC3	0.011							
	0.980							
RHC3	0.006	0.999						
	0.989	0.000						
TYPE	-0.520	0.021	0.053					
	0.187	0.960	0.900					
SEX	0.641	-0.390	-0.396	-0.577				
	0.086	0.339	0.331	0.134				
GP1	-0.249	0.268	0.263	-0.280	0.270			

TG	-0.167	-0.162	0.347	-0.220	-0.236	-0.049	0.548	-0.749
	0.692	0.702	0.399	0.601	0.574	0.908	0.160	0.033
TGR	-0.168	-0.163	0.350	-0.218	-0.235	-0.047	0.550	-0.746
	0.691	0.699	0.396	0.603	0.575	0.912	0.158	0.033
Z1MEAN	-0.430	-0.518	-0.538	-0.699	-0.693	-0.637	0.162	-0.323
	0.288	0.188	0.169	0.054	0.057	0.090	0.702	0.434
Z2MEAN	-0.886	-0.877	-0.767	-0.707	-0.685	-0.599	0.250	-0.219
	0.003	0.004	0.026	0.050	0.061	0.117	0.551	0.603
Z3MEAN	-0.817	-0.766	-0.544	-0.480	-0.463	-0.343	0.213	0.126
	0.013	0.027	0.163	0.229	0.248	0.405	0.613	0.767
Z4MEAN	0.027	-0.050	0.019	-0.176	-0.174	-0.138	0.402	0.414
	0.949	0.906	0.964	0.677	0.680	0.745	0.323	0.308
ZAFMAZ1	0.594	0.558	-0.005	-0.087	-0.099	-0.268	-0.338	-0.124
	0.120	0.150	0.991	0.837	0.816	0.521	0.413	0.770
ZAFMAZ2	-0.024	0.017	-0.505	-0.596	-0.576	-0.718	0.531	-0.114
	0.955	0.968	0.202	0.119	0.135	0.045	0.175	0.789
ZAFMAZ3	0.285	0.259	0.559	0.190	0.167	0.237	0.127	-0.060
	0.494	0.535	0.150	0.653	0.692	0.571	0.764	0.887
ZAFMAZ4	0.515	0.423	0.519	-0.222	-0.251	-0.187	0.423	-0.385
	0.191	0.297	0.188	0.598	0.549	0.657	0.297	0.346
ZAFTUZ1	0.494	0.438	0.796	0.814	0.797	0.818	-0.148	-0.081
	0.214	0.277	0.018	0.014	0.018	0.013	0.727	0.849
ZAFTUZ2	0.518	0.434	0.658	-0.056	-0.085	0.001	0.507	-0.265
	0.188	0.283	0.076	0.895	0.842	0.998	0.200	0.525
ZAFTUZ3	0.479	0.388	0.599	0.593	0.571	0.573	-0.409	0.177
	0.230	0.343	0.116	0.121	0.139	0.138	0.314	0.676
ZAFTUZ4	0.697	0.591	0.417	0.659	0.661	0.525	-0.057	0.527
	0.055	0.123	0.304	0.076	0.075	0.181	0.893	0.179
ZAFCOZ1	0.282	0.241	0.742	0.814	0.803	0.870	-0.024	-0.003
	0.499	0.565	0.035	0.014	0.016	0.005	0.956	0.993
ZAFCOZ2	0.431	0.336	0.823	0.196	0.167	0.327	0.509	-0.166
	0.286	0.416	0.012	0.643	0.692	0.429	0.197	0.695
ZAFCOZ3	0.413	0.323	0.536	0.584	0.563	0.565	-0.531	0.101
	0.309	0.435	0.171	0.128	0.146	0.144	0.176	0.813
ZAFCOZ4	0.657	0.558	0.372	0.694	0.699	0.554	-0.105	0.569
	0.077	0.150	0.365	0.056	0.054	0.154	0.804	0.141
ZAFITZ1	-0.494	-0.439	-0.796	-0.814	-0.797	-0.818	0.148	0.081
	0.213	0.277	0.018	0.014	0.018	0.013	0.727	0.849
ZAFITZ2	-0.420	-0.341	-0.626	0.040	0.061	-0.044	-0.715	0.216
	0.301	0.409	0.097	0.924	0.886	0.917	0.045	0.638
ZAFITZ3	-0.432	-0.341	-0.587	-0.697	-0.680	-0.681	0.428	-0.263
	0.285	0.408	0.126	0.055	0.064	0.063	0.290	0.530
ZAFITZ4	-0.736	-0.627	-0.458	-0.626	-0.624	-0.494	0.079	-0.475
	0.037	0.096	0.253	0.097	0.098	0.213	0.852	0.234

Fb 1	0.442	0.335	0.671	0.349	0.319	0.408	-0.149	-0.389
	0.273	0.418	0.069	0.397	0.441	0.315	0.725	0.340
Hb 1	0.226	0.102	0.774	0.457	0.431	0.631	0.111	-0.032
	0.591	0.809	0.024	0.255	0.286	0.093	0.793	0.941
Db 1	-0.197	-0.105	-0.044	-0.142	-0.147	-0.060	-0.031	0.339
	0.640	0.804	0.918	0.737	0.729	0.888	0.941	0.411
Fb 2	0.302	0.194	0.679	0.408	0.386	0.516	0.077	-0.320
	0.467	0.645	0.064	0.316	0.345	0.190	0.855	-0.440
Hb 2	0.287	0.176	0.760	0.385	0.360	0.539	0.207	-0.127
	0.491	0.676	0.029	0.347	0.381	0.168	0.622	0.764
Db 2	-0.386	-0.244	-0.663	-0.651	-0.645	-0.691	-0.225	-0.131
	0.345	0.561	0.073	0.080	0.084	0.058	0.593	0.757
Fb 3	0.369	0.263	0.750	0.460	0.434	0.563	-0.013	-0.299
	0.368	0.529	0.032	0.251	0.283	0.147	0.975	-0.473
H b3	0.242	0.134	0.739	0.305	0.282	0.475	0.348	-0.157
	0.564	0.752	0.036	0.463	0.499	0.235	0.399	0.710
D b3	-0.123	-0.002	-0.248	-0.472	-0.479	-0.463	-0.064	-0.004
	0.772	0.997	0.554	0.238	0.230	0.248	0.880	0.993

	RFC1	CFC2	RFC2	CFC3	RFC3	CHC1	RHC1	CHC2
CFC2	0.401							
	0.325							
RFC2	0.466	0.994						
	0.244	0.000						
CFC3	-0.285	-0.352	-0.353					
	0.494	0.393	0.392					
RFC3	-0.288	-0.335	-0.338	0.997				
	0.489	0.417	0.412	0.000				
CHC1	0.577	0.321	0.322	0.225	0.219			
	0.134	0.438	0.437	0.592	0.601			
RHC1	0.578	0.326	0.327	0.222	0.215	1.000		
	0.134	0.430	0.429	0.598	0.609	0.000		
CHC2	0.534	0.864	0.877	0.055	0.057	0.577	0.582	
	0.173	0.006	0.004	0.896	0.894	0.135	0.130	
RHC2	0.558	0.844	0.865	0.073	0.072	0.578	0.583	0.597
	0.151	0.008	0.006	0.864	0.866	0.134	0.129	0.000
CHC3	-0.166	-0.444	-0.417	0.943	0.935	0.244	0.238	-0.028
	0.695	0.270	0.304	0.000	0.001	0.561	0.571	0.948
RHC3	-0.154	-0.443	-0.414	0.934	0.929	0.245	0.239	-0.033
	0.716	0.272	0.307	0.001	0.001	0.559	0.570	0.938
TYPE	-0.011	-0.429	-0.408	-0.205	-0.186	-0.212	-0.223	-0.533
	0.980	0.288	0.315	0.626	0.660	0.613	0.596	0.174
SEX	0.070	0.818	0.784	-0.135	-0.105	0.164	0.170	0.696
	0.869	0.013	0.027	0.750	0.804	0.698	0.687	0.055
GP1	-0.659	-0.211	-0.255	0.481	0.513	-0.499	-0.502	-0.223
	0.076	0.616	0.541	0.228	0.193	0.208	0.205	0.596

	0.552	0.520	0.529	0.501	0.518			
GRP1	-0.250	0.266	0.261	-0.780	0.270	1.000		
	0.550	0.524	0.532	0.502	0.518	0.000		
GP2	-0.223	-0.184	-0.198	-0.297	0.343	0.772	0.772	
	0.596	0.662	0.639	0.475	0.406	0.025	0.025	
GRP2	-0.222	-0.182	-0.196	-0.297	0.343	0.774	0.774	1.000
	0.597	0.665	0.642	0.475	0.405	0.024	0.024	0.000
GP3	-0.716	-0.627	-0.615	0.503	-0.387	-0.181	-0.179	0.113
	0.046	0.096	0.104	0.204	0.343	0.668	0.672	0.790
GRP3	-0.716	-0.629	-0.617	0.504	-0.384	-0.179	-0.177	0.114
	0.046	0.095	0.103	0.203	0.348	0.672	0.676	0.788
GP4	-0.478	0.399	0.422	0.545	-0.674	-0.172	-0.172	-0.409
	0.231	0.328	0.297	0.163	0.067	0.684	0.685	0.314
GRP4	-0.478	0.399	0.423	0.545	-0.673	-0.171	-0.170	-0.409
	0.231	0.327	0.297	0.162	0.067	0.686	0.687	0.314
GP5	0.608	-0.276	-0.295	-0.849	0.898	0.361	0.361	0.478
	0.110	0.509	0.478	0.008	0.002	0.379	0.379	0.231
GRP5	0.608	-0.281	-0.300	-0.847	0.901	0.357	0.357	0.476
	0.109	0.501	0.470	0.008	0.002	0.385	0.385	0.234
TG	-0.560	-0.165	-0.162	-0.053	0.076	0.730	0.732	0.797
	0.149	0.697	0.701	0.901	0.857	0.040	0.039	0.018
TGR	-0.558	-0.167	-0.165	-0.054	0.077	0.726	0.728	0.794
	0.150	0.692	0.696	0.899	0.857	0.041	0.041	0.019
Z1MEAN	0.468	0.417	0.404	-0.476	0.515	0.309	0.308	0.120
	0.242	0.304	0.321	0.234	0.192	0.456	0.459	0.777
Z2MEAN	0.508	0.044	0.013	-0.966	0.606	0.357	0.356	0.404
	0.199	0.917	0.976	0.000	0.111	0.385	0.386	0.321
Z3MEAN	0.541	-0.105	-0.124	-0.849	0.420	0.128	0.128	0.273
	0.166	0.805	0.769	0.008	0.300	0.762	0.762	0.512
Z4MEAN	0.296	0.181	0.190	-0.251	-0.048	-0.326	-0.325	-0.546
	0.477	0.668	0.653	0.549	0.910	0.431	0.433	0.162
ZAFMAZ1	-0.140	0.628	0.633	0.497	-0.381	-0.112	-0.114	-0.394
	0.741	0.095	0.092	0.210	0.352	0.792	0.789	0.334
ZAFMAZ2	-0.097	0.586	0.566	-0.697	-0.147	0.368	0.368	-0.092
	0.819	0.127	0.144	0.210	0.729	0.369	0.370	0.829
ZAFMAZ3	-0.458	-0.223	-0.217	0.120	-0.507	-0.328	-0.327	-0.153
	0.254	0.596	0.606	0.705	0.200	0.427	0.430	0.717
ZAFMAZ4	-0.427	0.452	0.474	0.403	-0.359	0.209	0.209	-0.099
	0.279	0.261	0.235	0.323	0.382	0.619	0.619	0.815
ZAFTUZ1	-0.647	-0.651	-0.636	0.632	-0.265	-0.325	-0.323	-0.048
	0.103	0.080	0.090	0.093	0.526	0.433	0.436	0.911
ZAFTUZ2	-0.505	0.246	0.270	0.393	-0.403	0.117	0.118	-0.122
	0.202	0.558	0.517	0.335	0.322	0.783	0.780	0.773
ZAFTUZ3	-0.231	-0.377	-0.366	0.531	-0.394	-0.787	-0.786	-0.502
	0.581	0.357	0.372	0.206	0.334	0.020	0.021	0.205

ZAFTUZ4	-0.074	-0.091	-0.068	0.567	-0.205	-0.591	-0.590	-0.806
	0.861	0.831	0.873	0.143	0.627	0.123	0.124	0.016
ZAFCOZ1	-0.519	-0.815	-0.802	0.442	-0.110	-0.269	-0.267	0.064
	0.187	0.014	0.017	0.273	0.795	0.519	0.523	0.881
ZAFCOZ2	-0.410	-0.042	-0.006	0.517	-0.151	0.164	0.166	0.020
	0.313	0.921	0.990	0.189	0.721	0.697	0.694	0.963
ZAFCOZ3	-0.200	-0.410	-0.403	0.494	-0.327	-0.771	-0.771	0.422
	0.635	0.314	0.323	0.214	0.430	0.025	0.025	0.298
ZAFCOZ4	-0.035	-0.140	-0.119	0.538	-0.170	-0.621	-0.620	-0.806
	0.935	0.741	0.778	0.169	0.688	0.100	0.101	0.016
ZAFITZ1	0.617	0.651	0.636	-0.632	0.265	0.325	0.323	0.048
	0.103	0.080	0.090	0.093	0.526	0.433	0.436	0.910
ZAFITZ2	0.451	-0.187	-0.216	-0.322	0.208	-0.313	-0.315	0.010
	0.262	0.658	0.608	0.436	0.621	0.450	0.447	0.981
ZAFITZ3	0.172	0.496	0.484	-0.489	0.300	0.819	0.818	0.503
	0.684	0.212	0.224	0.219	0.470	0.013	0.013	0.204
ZAFITZ4	0.123	0.053	0.030	-0.595	0.281	0.616	0.615	0.817
	0.771	0.901	0.943	0.120	0.500	0.104	0.104	0.013
Fb 1	-0.377	-0.139	-0.115	0.725	-0.049	0.021	0.021	0.105
	0.358	0.743	0.787	0.042	0.909	0.961	0.960	0.805
Hb 1	-0.094	-0.390	-0.353	0.563	0.185	-0.067	-0.066	0.049
	0.824	0.339	0.390	0.146	0.660	0.874	0.877	0.908
Db 1	0.360	0.165	0.180	-0.019	-0.002	0.081	0.080	0.096
	0.381	0.696	0.669	0.965	0.997	0.849	0.850	0.821
Fb 2	-0.318	-0.297	-0.269	0.621	0.170	0.170	0.171	0.224
	0.443	0.475	0.519	0.100	0.688	0.687	0.685	0.594
Hb 2	-0.188	-0.261	-0.223	0.605	0.160	0.113	0.115	0.123
	0.656	0.532	0.595	0.112	0.706	0.789	0.787	0.771
Db 2	0.291	0.546	0.526	-0.370	-0.020	0.369	0.367	0.328
	0.484	0.162	0.180	0.367	0.962	0.368	0.372	0.428
Fb 3	-0.354	-0.311	-0.282	0.695	0.067	0.064	0.065	0.167
	0.390	0.453	0.499	0.056	0.875	0.881	0.879	0.693
H b3	-0.201	-0.229	-0.192	0.525	0.186	0.212	0.214	0.173
	0.633	0.585	0.649	0.182	0.659	0.613	0.611	0.682
D b3	0.195	0.561	0.566	-0.024	-0.144	0.364	0.362	0.238
	0.644	0.148	0.144	0.955	0.734	0.376	0.378	0.571

	GRP2	GP3	GRP3	GP4	GRP4	GP5	GRP5	TG
GP3	0.111							
	0.794							
GRP3	0.112	1.000						
	0.791	0.000						
GP4	-0.411	0.255	0.254					
	0.311	0.542	0.544					
GRP4	-0.411	0.255	0.254	1.000				
	0.311	0.542	0.544	0.000				

GP5	0.478	-0.435	-0.433	-0.660	-0.660			
	0.230	0.282	0.284	0.075	0.075			
GRP5	0.476	-0.432	-0.431	-0.662	-0.662	1.000		
	0.234	0.285	0.286	0.074	0.074	0.000		
TG	0.796	0.414	0.415	0.128	0.129	0.198	0.195	
	0.018	0.308	0.306	0.762	0.762	0.638	0.643	
TGR	0.792	0.415	0.417	0.132	0.132	0.199	0.197	1.000
	0.019	0.306	0.304	0.756	0.755	0.636	0.641	0.000
Z1MEAN	0.120	-0.778	-0.776	-0.088	-0.088	0.555	0.554	-0.020
	0.776	0.023	0.024	0.836	0.835	0.153	0.154	0.962
Z2MEAN	0.404	-0.554	-0.554	-0.482	-0.483	0.879	0.877	0.152
	0.321	0.154	0.154	0.226	0.225	0.004	0.004	0.719
Z3MEAN	0.273	-0.312	-0.314	-0.396	-0.397	0.720	0.717	-0.096
	0.514	0.452	0.449	0.331	0.331	0.044	0.045	0.821
Z4MEAN	-0.548	-0.187	-0.189	0.518	0.518	0.071	0.070	-0.179
	0.160	0.657	0.654	0.188	0.188	0.867	0.870	0.671
ZAFMAZ1	-0.392	-0.331	-0.330	0.354	0.354	-0.541	-0.540	-0.401
	0.336	0.424	0.425	0.390	0.390	0.167	0.167	0.325
ZAFMAZ2	-0.090	-0.405	-0.406	0.160	0.161	0.096	0.093	0.041
	0.831	0.319	0.318	0.704	0.703	0.821	0.827	0.923
ZAFMAZ3	-0.157	0.637	0.634	0.644	0.643	-0.323	-0.324	0.301
	0.711	0.090	0.091	0.085	0.085	0.436	0.434	0.468
ZAFMAZ4	-0.101	0.073	0.074	0.879	0.879	-0.374	-0.376	0.387
	0.812	0.864	0.862	0.004	0.004	0.362	0.359	0.343
ZAFTUZ1	-0.050	0.898	0.900	0.250	0.249	-0.451	-0.447	0.224
	0.907	0.002	0.002	0.551	0.552	0.262	0.267	0.593
ZAFTUZ2	-0.125	0.304	0.304	0.911	0.911	-0.389	-0.391	0.458
	0.768	0.464	0.464	0.002	0.002	0.341	0.338	0.254
ZAFTUZ3	-0.505	0.556	0.555	0.494	0.492	-0.479	-0.475	-0.222
	0.202	0.152	0.153	0.214	0.216	0.229	0.234	0.597
ZAFTUZ4	-0.806	0.183	0.185	0.396	0.396	-0.519	-0.514	-0.516
	0.016	0.664	0.662	0.332	0.331	0.187	0.193	0.191
ZAFCOZ1	0.062	0.937	0.938	0.101	0.100	-0.252	-0.247	0.314
	0.885	0.001	0.001	0.813	0.813	0.547	0.555	0.448
ZAFCOZ2	0.017	0.424	0.426	0.700	0.701	-0.284	-0.285	0.530
	0.967	0.295	0.293	0.053	0.053	0.495	0.494	0.177
ZAFCOZ3	-0.424	0.519	0.519	0.373	0.371	-0.429	-0.425	-0.240
	0.295	0.187	0.188	0.362	0.365	0.289	0.294	0.568
ZAFCOZ4	-0.806	0.180	0.181	0.310	0.311	-0.490	-0.484	-0.563
	0.016	0.670	0.667	0.455	0.454	0.218	0.224	0.146
ZAFITZ1	0.050	-0.898	-0.900	-0.250	-0.249	0.452	0.447	-0.224
	0.907	0.002	0.002	0.551	0.552	0.261	0.267	0.593
ZAFITZ2	0.012	-0.267	-0.268	-0.763	-0.764	0.245	0.247	-0.552
	0.977	0.523	0.520	0.028	0.027	0.558	0.555	0.156

ZAFITZ3	0.506	-0.581	-0.580	-0.373	-0.371	0.426	0.421	0.267
	0.201	0.131	0.132	0.363	0.365	0.292	0.298	0.523
ZAFITZ4	0.817	-0.209	-0.210	-0.488	-0.488	0.569	0.564	0.491
	0.013	0.620	0.618	0.220	0.219	0.141	0.146	0.217
Fb 1	0.104	0.335	0.339	0.335	0.335	-0.338	-0.335	0.259
	0.806	0.417	0.412	0.417	0.417	0.413	0.418	0.536
Hb 1	0.047	0.394	0.397	0.309	0.309	-0.102	-0.100	0.299
	0.912	0.334	0.330	0.457	0.456	0.809	0.814	0.472
Db 1	0.096	-0.181	-0.183	-0.125	-0.124	0.043	0.039	-0.056
	0.820	0.668	0.665	0.768	0.770	0.919	0.928	0.895
Fb 2	0.223	0.371	0.375	0.189	0.190	-0.155	-0.152	0.386
	0.595	0.366	0.360	0.654	0.653	0.714	0.720	0.345
Hb 2	0.122	0.346	0.350	0.320	0.321	-0.144	-0.142	0.376
	0.774	0.401	0.396	0.439	0.438	0.733	0.737	0.358
Db 2	0.330	-0.579	-0.581	-0.398	-0.398	0.187	0.181	-0.104
	0.425	0.133	0.131	0.329	0.329	0.658	0.668	0.806
Fb 3	0.166	0.441	0.445	0.262	0.262	-0.248	-0.245	0.351
	0.695	0.274	0.269	0.531	0.530	0.553	0.558	0.394
H b3	0.171	0.328	0.331	0.343	0.345	-0.084	-0.083	0.466
	0.685	0.428	0.423	0.405	0.403	0.843	0.845	0.245
D b3	0.239	-0.405	-0.407	-0.094	-0.093	-0.043	-0.049	0.005
	0.569	0.320	0.317	0.824	0.826	0.919	0.907	0.992

	TGR	Z1MEAN	Z2MEAN	Z3MEAN	Z4MEAN	ZAFMAZ1	ZAFMAZ2	ZAFMAZ3
Z1MEAN	-0.020							
	0.962							
Z2MEAN	0.153	0.632						
	0.717	0.093						
Z3MEAN	0.099	0.173	0.805					
	0.815	0.682	0.016					
Z4MEAN	-0.172	0.194	0.214	0.385				
	0.684	0.646	0.610	0.346				
ZAFMAZ1	-0.405	0.320	-0.408	-0.740	-0.183			
	0.320	0.439	0.316	0.036	0.664			
ZAFMAZ2	0.041	0.337	0.437	0.235	0.378	0.117		
	0.923	0.415	0.278	0.575	0.359	0.782		
ZAFMAZ3	0.308	-0.395	-0.169	0.098	0.521	-0.335	0.013	
	0.459	0.332	0.689	0.817	0.185	0.418	0.975	
ZAFMAZ4	0.390	0.257	-0.264	-0.387	0.393	0.384	0.217	0.408
	0.340	0.538	0.527	0.343	0.335	0.347	0.606	0.316
ZAFTUZ1	0.226	-0.573	-0.643	-0.575	-0.243	-0.046	-0.528	0.468
	0.591	0.137	0.085	0.136	0.562	0.914	0.179	0.242
ZAFTUZ2	0.462	0.019	-0.306	-0.274	0.506	0.124	0.171	0.637
	0.250	0.965	0.462	0.512	0.201	0.770	0.685	0.089
ZAFTUZ3	-0.217	-0.310	-0.486	-0.370	0.262	0.122	-0.429	0.659
	0.605	0.455	0.222	0.367	0.530	0.773	0.289	0.076

ZAFTUZ4	-0.514	-0.163	-0.638	-0.663	0.241	0.464	-0.124	0.075
	0.193	0.699	0.089	0.073	0.566	0.247	0.771	0.860
ZAFCOZ1	0.317	-0.647	-0.489	-0.319	-0.182	-0.353	-0.535	0.508
	0.445	0.083	0.219	0.441	0.667	0.390	0.172	0.198
ZAFCOZ2	0.533	-0.121	-0.435	-0.312	0.302	-0.047	-0.200	0.462
	0.174	0.775	0.282	0.452	0.467	0.912	0.634	0.249
ZAFCOZ3	-0.236	-0.250	-0.456	-0.407	0.112	0.178	-0.508	0.544
	0.574	0.550	0.256	0.316	0.792	0.674	0.199	0.163
ZAFCOZ4	-0.561	-0.189	-0.623	-0.642	0.197	0.438	-0.150	0.031
	0.148	0.654	0.099	0.086	0.640	0.277	0.723	0.942
ZAFITZ1	-0.226	0.573	0.644	0.576	0.243	0.045	0.528	-0.467
	0.591	0.138	0.085	0.135	0.561	0.915	0.179	0.243
ZAFITZ2	-0.555	-0.011	0.262	0.219	-0.447	0.003	-0.191	-0.468
	0.153	0.979	0.531	0.603	0.267	0.995	0.651	0.242
ZAFITZ3	0.262	0.362	0.496	0.361	-0.205	-0.060	0.506	-0.587
	0.531	0.378	0.211	0.379	0.627	0.888	0.200	0.126
ZAFITZ4	0.488	0.154	0.649	0.675	-0.284	-0.492	0.113	-0.165
	0.220	0.716	0.081	0.066	0.495	0.216	0.791	0.696
Fb 1	0.258	0.060	-0.561	-0.760	-0.352	0.448	-0.568	-0.007
	0.537	0.889	0.148	0.029	0.393	0.266	0.141	0.987
Hb 1	0.301	-0.089	-0.458	-0.364	0.014	-0.053	-0.698	0.171
	0.468	0.835	0.254	0.376	0.974	0.901	0.054	0.686
Db 1	-0.057	-0.277	-0.021	0.433	0.051	-0.340	-0.229	-0.141
	0.893	0.506	0.960	0.284	0.904	0.410	0.585	0.739
Fb 2	0.385	0.012	-0.491	-0.640	-0.355	0.215	-0.586	-0.080
	0.346	0.978	0.217	0.087	0.388	0.609	0.127	0.851
Hb 2	0.377	-0.055	-0.493	-0.458	-0.086	0.047	-0.602	0.055
	0.357	0.896	0.214	0.254	0.840	0.913	0.114	0.898
Db 2	-0.109	0.171	0.382	0.403	-0.294	0.057	0.250	-0.493
	0.797	0.685	0.351	0.322	0.479	0.894	0.550	0.215
Fb 3	0.350	-0.062	-0.564	-0.667	-0.331	0.228	-0.646	0.019
	0.395	0.885	0.146	0.072	0.423	0.587	0.084	0.965
H b3	0.467	-0.026	-0.417	-0.377	-0.016	-0.027	-0.502	0.088
	0.243	0.951	0.304	0.355	0.971	0.950	0.205	0.837
D b3	0.000	-0.036	0.041	0.260	-0.168	0.027	0.046	-0.356
	0.999	0.932	0.922	0.533	0.691	0.949	0.913	0.387

	ZAFMAZ4	ZAFTUZ1	ZAFTUZ2	ZAFTUZ3	ZAFTUZ4	ZAFCOZ1	ZAFCOZ2	ZAFCOZ3
ZAFTUZ1	0.125							
	0.768							
ZAFTUZ2	0.944	0.270						
	0.000	0.518						
ZAFTUZ3	0.224	0.712	0.335					
	0.593	0.048	0.417					
ZAFTUZ4	0.232	0.468	0.242	0.576				
	0.581	0.242	0.564	0.135				

ZAFCOZ1	-0.025	0.949	0.192	0.610	0.317			
	0.953	0.000	0.649	0.108	0.444			
ZAFCOZ2	0.788	0.413	0.874	0.272	0.255	0.394		
	0.020	0.309	0.005	0.515	0.542	0.334		
ZAFCOZ3	0.140	0.719	0.212	0.981	0.531	0.599	0.171	
	0.741	0.045	0.615	0.000	0.175	0.117	0.686	
ZAFCOZ4	0.134	0.468	0.147	0.565	0.995	0.328	0.177	0.530
	0.752	0.242	0.728	0.145	0.000	0.428	0.675	0.177
ZAFITZ1	-0.125	-1.000	-0.270	-0.712	-0.468	-0.949	-0.413	-0.719
	0.768	0.000	0.518	0.048	0.242	0.000	0.309	0.045
ZAFITZ2	-0.875	-0.207	-0.943	-0.102	-0.202	-0.186	-0.924	0.030
	0.004	0.623	0.000	0.810	0.632	0.660	0.001	0.944
ZAFITZ3	-0.099	-0.751	-0.226	-0.986	-0.610	-0.673	-0.224	-0.975
	0.815	0.032	0.591	0.000	0.108	0.068	0.594	0.000
ZAFITZ4	-0.310	-0.488	-0.321	-0.645	-0.992	-0.321	-0.295	-0.595
	0.454	0.220	0.439	0.084	0.000	0.438	0.479	0.120
Fb 1	0.499	0.605	0.395	0.401	0.368	0.423	0.577	0.468
	0.208	0.112	0.333	0.324	0.370	0.296	0.134	0.242
Hb 1	0.420	0.542	0.465	0.388	0.323	0.527	0.792	0.379
	0.301	0.165	0.245	0.343	0.435	0.179	0.019	0.354
Db 1	-0.221	-0.439	-0.158	-0.394	-0.402	-0.295	0.039	-0.441
	0.599	0.277	0.708	0.334	0.323	0.478	0.927	0.274
Fb 2	0.413	0.597	0.357	0.235	0.317	0.499	0.654	0.286
	0.310	0.118	0.385	0.575	0.444	0.208	0.079	0.493
Hb 2	0.490	0.489	0.497	0.232	0.300	0.451	0.825	0.225
	0.217	0.219	0.210	0.581	0.471	0.262	0.012	0.592
Db 2	-0.356	-0.768	-0.492	-0.707	-0.690	-0.740	-0.564	-0.654
	0.386	0.026	0.216	0.050	0.058	0.036	0.145	0.078
Fb 3	0.430	0.662	0.390	0.354	0.347	0.552	0.670	0.401
	0.288	0.074	0.339	0.390	0.399	0.156	0.069	0.325
H b3	0.540	0.429	0.562	0.151	0.237	0.416	0.874	0.126
	0.167	0.289	0.147	0.720	0.571	0.305	0.005	0.766
D b3	-0.083	-0.633	-0.163	-0.626	-0.536	-0.598	-0.106	-0.633
	0.845	0.092	0.700	0.097	0.171	0.117	0.803	0.092

	ZAFCOZ4	ZAFITZ1	ZAFITZ2	ZAFITZ3	ZAFITZ4	Fb 1	Hb 1	Db 1
ZAFITZ1	-0.468							
	0.242							
ZAFITZ2	-0.113	0.207						
	0.790	0.623						
ZAFITZ3	-0.612	0.750	0.024					
	0.107	0.032	0.956					
ZAFITZ4	-0.979	0.489	0.246	0.660				
	0.000	0.219	0.557	0.075				
Fb 1	0.330	-0.605	-0.356	-0.391	-0.394			
	0.424	0.112	0.387	0.338	0.334			

Hb 1	0.287	-0.542	-0.520	-0.414	-0.330	0.779		
	0.490	0.165	0.186	0.308	0.425	0.023		
Db 1	-0.395	0.439	0.051	0.376	0.429	-0.331	0.050	
	0.333	0.277	0.905	0.358	0.289	0.423	0.906	
Fb 2	0.287	-0.597	-0.426	-0.260	-0.312	0.940	0.869	-0.229
	0.491	0.118	0.292	0.534	0.452	0.001	0.005	0.585
Hb 2	0.257	-0.489	-0.591	-0.250	-0.299	0.820	0.972	0.051
	0.540	0.218	0.123	0.550	0.472	0.013	0.000	0.904
Db 2	-0.667	0.768	0.465	0.734	0.707	-0.493	-0.605	0.570
	0.071	0.026	0.245	0.038	0.050	0.215	0.112	0.140
Fb 3	0.315	-0.662	-0.420	-0.372	-0.355	0.962	0.889	-0.220
	0.447	0.074	0.301	0.365	0.388	0.000	0.003	0.601
H b3	0.187	-0.429	-0.680	-0.162	-0.236	0.754	0.948	0.075
	0.657	0.289	0.064	0.701	0.573	0.031	0.000	0.861
D b3	-0.543	0.633	0.086	0.658	0.550	-0.278	-0.206	0.847
	0.165	0.092	0.839	0.076	0.158	0.505	0.625	0.008

	Fb 2	Hb 2	Db 2	Fb 3	H b3
Hb 2	0.923				
	0.001				
Db 2	-0.535	-0.527			
	0.172	0.179			
Fb 3	0.989	0.922	-0.564		
	0.000	0.001	0.146		
H b3	0.885	0.988	-0.514	0.873	
	0.003	0.000	0.193	0.005	
D b3	-0.266	-0.112	0.846	-0.278	-0.087
	0.524	0.792	0.008	0.505	0.838

Cell Contents: Correlation
P-Value

APPENDIX V

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Tubule density of the *stratum medium* of horse hoof

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Keywords: horse; Tubule density; *stratum medium*; hoof

Summary

The number of tubules/mm^2 (tubule density) of horse hoof horn was quantified in samples taken from the left forefeet of 8 randomly selected slaughterhouse horses in order to establish the normal tubule density characteristics at the midline dead centre (MDC) for the *stratum medium* of horse hoof. In the past the measurement of tubule distribution within the hoof has lacked objectivity. The horse hoof tubule density results are compared to a recent objective study carried out on pony hoof. A similar 4 zone pattern of tubule density was observed, although the precise zonal boundaries and tubule density values differed to those found for pony hoof. There were significant differences in tubule density between zones. Comparison with pony hoof revealed significant tubule density differences in zones 1, 2 and 4; however, there was no significant difference in zone 3. The existence of a 4 zoned pattern of tubule density for horse hoof, as for pony hoof, has been confirmed.

Introduction

Reilly *et al.* (1996) and Kasapi and Gosline (1997) noted that the hoof wall exhibits a structural hierarchy evident at the macroscopic, microscopic and ultrastructural level. At the macroscopic level, hoof wall is composed of 3 morphologically distinct layers (Trautmann and Feibiger 1957), namely the *stratum internum*, *stratum medium* and *stratum externum*. The *stratum medium*, which constitutes the bulk of the hoof wall (Nickel 1938, 1939), forms the basis of this study and is considered to be the principle component determining the load bearing capabilities of the hoof wall (Pollitt 1992; Kasapi and Gosline 1997). At the microscopic level the *stratum medium* exhibits a distinctive structural architecture composed of tubular and intertubular horn (Bruhnke 1931; Nickel 1938, 1939; Wilkens 1964).

Horn tubules are composed of a central medullary cavity, or marrow, surrounded by a cellular cortex. The individual horn tubules are surrounded and separated by intertubular horn. These horn tubules are assumed to be continuous from the coronary band to the bearing border (Pollitt 1995) and, in cattle, are said to be arranged parallel to the wall (Greenough *et al.* 1981). The hoof wall derives strength and form from the orderly proximodistal arrangements of tubular and intertubular horn (Balch *et al.* 1997). It is believed that this structural arrangement is responsible for the functional capacity of the hoof (Nickel 1939; Schummer *et al.* 1981) and the distribution of forces within the capsule (Bertram and Gosline 1987).

Tubule density of the *stratum medium* has been defined as the number of tubules/unit area (Reilly *et al.* 1996) and many workers have suggested that this parameter influences the macroscopic mechanical properties of hoof horn. For example, Gunther *et al.* (1983) and Geyer and Tagwerker (1986) for cattle and pig hoof, respectively, suggested that 'hardness' was related to tubule density. In an early study, Bruhnke (1931) stated that the tensile/shear force of the horn of cattle, sheep, pigs and horses decreased from the dorsal aspect towards the palmar/plantar aspect. Much later, Wilkens (1964) reported that the mechanical strength of the hoof wall was dependent upon tubule density. However, no data were given to support this statement. More recently, Geyer (1980) summarised the work of Kind (1961) as suggesting that the higher the tubular density the greater the loading to which the horn may be subjected without compromise. Schummer *et al.* (1981) suggested a relationship between tubule density and resistance to wear of the hoof horn capsule. Dittrich *et al.* (1994) believed that an increase in the number of tubules improved hoof horn integrity, although again no data were presented to support this argument. The precise nature of the implied relationship between tubule density and the mechanical properties of hoof still needs to be evaluated.

Reilly *et al.* (1996) suggested that a specific pattern of tubule density may exist which reflects the functional demands placed on equine hoof. A variation in tubule density in different locations around the equine hoof capsule has been described by Lungwitz and Adams (1913), Nickel (1938, 1939), Geyer (1980), Bolliger (1991), Pellman *et al.* (1993), Bragulla *et al.* (1994) and Reilly *et al.* (1996). Differences also occur across the depth of the hoof wall (Nickel 1938, 1939; Zoerb and Leach 1978; Reilly *et al.* 1996; Kasapi and Gosline 1997) and descriptions of morphological zonation within the hoof wall have been reported for the horse (Bruhnke 1931; Nickel 1938, 1939; Wilkens 1964; Stump 1967; Leach 1980; Bolliger 1991).

Reilly (1995) argued that there is a need to measure objectively hoof horn parameters and consequently Reilly *et al.* (1996) established a systematic method and provided a detailed protocol for ascertaining tubule density in pony hoof. This provided a quantitative and objective measurement of this anatomical feature within the hoof capsule. The existence of 4 separate zones of hoof horn within the *stratum medium* at the midline dead centre (MDC) was proposed by Reilly *et al.* (1996) (Fig 1); and it was further suggested that this may be an equine pattern.

The aim of this study was to establish the normal tubule density for the *stratum medium* of the hoof of the horse, and to compare the zonal arrangement of tubules reported by Reilly *et*

TABLE 1: Comparison of tubule density and zonation in the *stratum medium* at the midline dead centre (MDC) for horse and pony hoof

	Horse		Pony (Reilly *et al.* 1996)	
Zone	% HWD	Tubule density (tubules/mm^2)	% HWD	Tubule density (tubules/mm^2)
Zone 1	0–25	>22	0–26	>27
Zone 2	25–47	16–22	26–51	16–27
Zone 3	47–69	11–16	51–77	8–16
Zone 4	69–100	<11	77–100	<8

HWD = hoof wall depth.

al. (1996) from a selected and controlled population of ponies, to the *stratum medium* obtained from a randomly selected slaughterhouse population of horses.

Materials and methods

The left fore feet of 8 randomly selected slaughterhouse horses were used to provide hoof samples. The sampling site was the midline dead centre of the *stratum medium* of the hoof wall after Reilly *et al.* (1996).

The centre point of the sample was taken as the mid point between the coronary band and the bearing border. The final block taken from the hoof capsule was 1 cm in height, 1 cm long (mediolaterally) and encompassed the full dorso-palmar extent of the stratum medium of the hoof wall. Reilly *et al.* (1996) referred to this as the full hoof wall depth of the *stratum medium* (HWD). The sample was taken at right angles to the direction of the tubules. A sledge microtome was used to cut a horizontal section of 12 µm from the centre of the block. Sections were stained in Haematoxylin and Eosin, dehydrated and mounted in DPX with a coverslip. Digitised images of each section were captured using a video camera and Global Laboratory software. Images were processed using NIH Image (Public domain software version 1.59) to enhance tubule definition. The digitised images were enlarged using Adobe Photoshop[1] to A4 format, prior to printing. A grid was then overlaid on the image and a tubule density count was carried out according to the method of Reilly *et al.* (1996).

Statistical analysis

The results were analysed using Minitab[2]. Graphs were produced using Minitab and Excel[3]. The normality of data was established using the Kolmogorov-Smirnov normality test. Differences between zones for transformed data were analysed using one way analysis of variance (ANOVA) and Tukey test. Zonal tubule density comparisons between horse and pony were evaluated by *t* test using transformed data.

Results

The frequency histograms for nontransformed and transformed horse tubule density data are shown in Figures 2a and 2b. The distribution in Figure 2a is skewed to the right such that the mean and median values are not the same. The normal probability plot indicated that the data were non-normally distributed ($P<0.01$). Square root (sqrt) transformation of the data after Reilly *et al.* (1996) gave a distribution that was normal ($P>0.01$) by the Kolmogorov-Smirnov normality test (Fig 2b).

TABLE 2: Mean and range tubule density values for equine hoof (tubules/mm^2)

Author	Tubule density
Kind (1961)	(5–9)
Leach (1980)	30
Bucher (1987)	8 and 14
Pellman *et al.* (1993)	7
Reilly *et al.* (1996)	16 (3–61)
Kasapi and Gosline (1997)	(10–25)
Reilly *et al.* (1998)	15 (5–48)

The results for tubule density as a function of percentage hoof wall depth (%HWD) are illustrated in Figure 3. Subsequent analysis of the data follows the convention given by Reilly *et al.* (1996). Dividing the transformed data, by use of ± 1 and ± 2 s.d. about the mean (3.95 sqrt tubules/mm^2), gave divisions at 2.56, 3.26, 4.65 and 5.34 sqrt tubules/mm^2 which correspond to tubule densities of ~6.6, ~10.6, ~21.6 and ~28.5 tubules/mm^2, with a mean of ~15.6. The square root tubule density values, at conventional standard deviations about the mean, delimit the zonal boundaries. These values were used to establish the %HWD at which the zonal boundaries occurred. A simple regression equation defined the relationship between %HWD and sqrt tubule/mm^2 as:-

$$\%HWD = 172 - 31.7 \text{ sqrt tubule density}$$

This equation was used to determine the corresponding intercepts in terms of percentage hoof wall depth for each square root tubule density value.

In this way the *stratum medium* of the hoof wall at the MDC was divided into 4 zones at ~25%, ~47% and ~69% HWD. The corresponding tubule density ranges are shown in Table 1.

There were significant differences in transformed tubule density values between all zones ($P<0.05$ by one-way ANOVA and Tukey test).

In addition, zones 1 and 2 tubule density values for the horse were significantly lower than those for the pony ($P<0.05$), whereas those for zone 4 were significantly higher ($P<0.01$). There was no significant difference in zone 3 for tubule density values between horse and pony horn ($P>0.05$).

Discussion

The results reported in this paper resemble those obtained by Reilly *et al.* (1996) from a pony population and have been compared in Table 1. The dorso-palmar decrease in tubule density is not uniform across the hoof wall and a distinct zonal pattern is evident (Fig 3).

Although differing tubule densities occur in the different zones of the *stratum medium*, the mean tubule density based upon transformed data is the same for both horses and ponies (~16 tubules/mm^2). This may represent an optimal mean tubule density for this site within the hoof capsule. Although the zonal trends are similar in both cases, the absolute distribution of tubule density within the zones varies.

The scatter plot (Fig 3) reveals 2 regions of changing tubule density (zones 1 and 3) and one region of relatively consistent tubule density (zone 2). This is similar to the findings of Reilly *et al.* (1996). The tubule density trend in zone 4, however, was difficult to ascertain. Excessive take up of stain resulted in 27

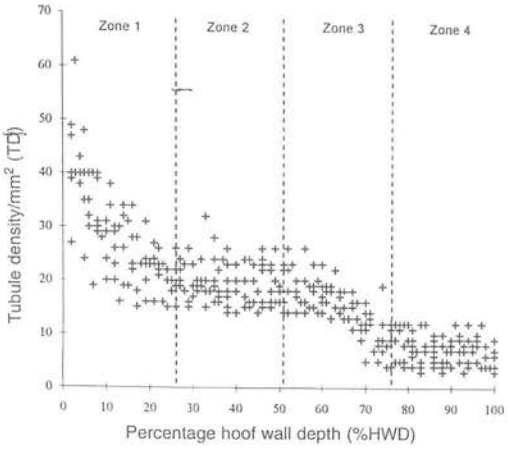


Fig 1: Tubule density (TD) by percentage hoof wall depth (%HWD) to show the 4 zones of the stratum medium *at the midline dead centre for ponies (after Reilly* et al. 1996).

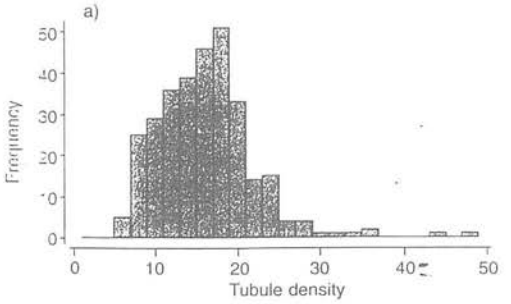
incomplete cell counts from a total of 308. This occurred solely in zone 4. However, Reilly *et al.* (1996) successfully completed a tubule density count from this region and showed consistent tubule density values in this zone giving a similar pattern, but different objective values, to that seen in zone 2.

The adjusted means and scatter plots for both horse and pony tubule density indicate a significantly lower tubule density in zones 1 and 2 for horse *stratum medium* ($P<0.01$). This may represent a functional difference between horse and pony horn. However, this difference in tubule density may relate to the method of image analysis employed. It can be argued that a video based system cannot match the resolving power of conventional optics and photography. This may not have allowed very small tubules to be resolved. Another reason for the difference may be the use of slaughterhouse samples which may have been biased towards older animals. While the precise relationship between tubule density and age for horses is not known, both Gunther (1974) and Geyer (1980) have suggested that in cloven hooves the number of tubules diminishes with age. If a similar relationship exists in the horse, lower tubule density values could be anticipated if such a sampling bias occurred in this study.

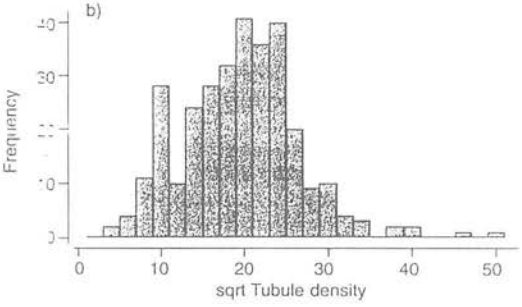
The tubule density values within zone 3 for horse and pony (11–16/mm^2 and 8–16/mm^2 respectively) were not significantly different ($P>0.05$). However, significant differences occurred in zone 4 (<11/mm^2, <8/mm^2, horse and pony respectively). These may represent differences in optimal tubule density values in this zone between horse and pony respectively.

The boundaries of zones 1 and 2 are close to those determined by Reilly *et al.* (1996). Determination of the boundary between zones 3 and 4 based upon a simple regression line indicates a boundary at ~68% of the hoof wall depth for our horses compared with ~77% for the ponies. This may reflect a genuine difference between horse and pony hoof or may represent the influence of excessive uptake of stain in zone 4. The latter may contribute to the disparity in tubule density values between other authors. Under representation of the tubule density contribution from zone 4 would cause a shift to the left of the zonal boundaries on the scatter plot (Fig 3), with the shift at the zone 3/4 boundary being more pronounced.

Previously published tubule density values for horse hoof



Count	Mean	Median	s.d.	Kurtosis
308	16.095	15.726	5.800	4.557



Count	Mean	Median	s.d.	Kurtosis
308	3.951	3.966	0.696	1.290

Fig 2: Frequency histograms for horse tubule density data: a) non transformed data and b) square root transformed data.

are shown in Table 2. These results show considerable variation between studies with several values differing from those reported in this study. However, direct comparisons are made difficult as details of methodologies and sampling areas have not been published by previous authors.

Reilly *et al.* (1996) demonstrated the potential overestimation of tubule density values if working with non-normal data. The tubule density data in this study displays a non-normal distribution which is consistent with that reported in the pony. Hence the true mean of tubular density should be calculated from the transformed data. In this case, the true mean of $(3.9513)^2$ tubules/mm^2 corresponds to 15.6 tubules/mm^2, which contrasts with a 'mean' of 16.1 tubules/mm^2 based upon untransformed data. This discrepancy highlights the slight overestimation that can occur if non transformed data is used for analysis. Reilly *et al.* (1996) emphasised the importance of this overestimation when interpreting work from different authors; and when subsequently relating tubule density to other physical properties.

A four zone division of the *stratum medium* of the hoof wall is proposed for the horse with an adjusted mean of >22, 16–22, 11–16 and <11 tubules/mm^2. This is in contrast to Bucher (1987) who divided the *stratum medium* into an 'inner' and 'outer' zone without clearly defining zonal boundaries. The tubule density of

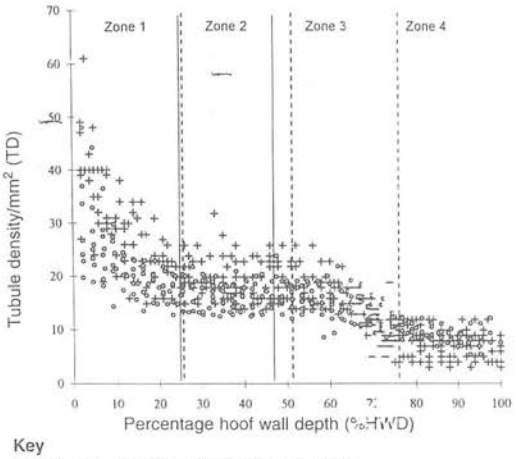


Fig 3: Comparison of tubule density (TD) and zonal boundaries by percentage hoof wall depth (%HWD) for the stratum medium *of ponies and horses.*

Bucher's (1987) 'inner' zone (8 tubules/mm^2) is slightly lower than that for zone 4 in this study and is similar to the results of Reilly *et al.* (1996) for pony hoof. The results from the outer zone from Bucher's study (14 tubules/mm^2) were considerably lower than either zone 1 or 2 for the pony hoof studies (>27/mm^2) by Reilly *et al.* (1996), or for horse hoof (>22/mm^2). The lower tubule density reported by Bucher (1987) may have arisen because the sample set included the *stratum medium* from hind feet. Both Reilly *et al.* (1996) and this study used left forefeet only. The relationship for tubule density and %HWD for hind feet is not known. The sample site used by Bucher (1987) also differed from that in this study.

A detailed methodology was not provided by Kasapi and Gosline (1997) when assessing the tubule density of the right fore hoof for a limited sample population of only 2 horses. They reported a palmaro-dorsal increase in tubule density across the hoof wall from 10 tubules/mm^2 in the 'inner wall' to 25 tubules /mm^2 towards the 'outer wall' which they refer to as a 'radial increase'. It is not possible to see whether a zonal pattern exists in their results, as only 6 equidistant sampling sites were selected. Nevertheless, the results are in broad agreement with this paper and those reported by Reilly *et al.* (1996).

The *stratum medium* of horse hoof has previously been divided into 3 zones ('outer', 'middle' and 'inner') based upon tubular morphology (Bruhnke 1931; Nickel 1938, 1939; Wilkens 1964). Stump (1967) agreed with this description, but also reported an 'intermediate' zone between the 'inner' and 'middle' zones containing tubule forms from both. Kasapi and Gosline (1997) found abrupt morphological changes in tubule form at 66% of the hoof wall depth which they interpreted as being the 'intermediate' zone first described by Stump (1967) and reported by Leach (1980). This area corresponds to zone 3 in this study. The transition between the low tubule density of zone 4 and the higher tubule density of zone 2 may account for the progressive change across zone 3.

Another population of tubules is reported to exist between zone 4 and the *stratum internum*. This has been described by Leach (1980), Bucher (1987), Bolliger (1991), Bragulla *et al.* (1994), Budras and Huskamp (1994) and Reilly *et al.* (1996). However, it is unclear whether this population is part of the *stratum medium* or is part of the cap horn tubule population. These tubules were not recorded as a separate entity in this study, either because their density made little impact upon the density values for zone 4 or because they fell outside the sampling area due to the curvature of the *stratum internum*. The method used in this investigation may therefore underestimate the true mean tubule density (see Reilly *et al.* 1996).

While the precise functional significance of zonal tubule density variation has yet to be fully investigated, it is possible that such variation will confer differences in mechanical properties (Reilly *et al.* 1996), modulated by moisture content (Bertram and Gosline 1987), and commensurate with the differential mechanical demands required across the wall (Kasapi and Gosline 1997). The hoof capsule plays a significant part in dampening concussive locomotory forces (Dyhre-Poulsen *et al.* 1994) and, in terms of athleticism, the horse represents a pinnacle of evolution (Bolliger 1991; Reilly *et al.* 1996). A compromise must exist between the need to accommodate forces, minimise crack propagation and avoid excessive deformation that would threaten dermal integrity. Vogel (1989) stated that the biomechanical properties of a structure are probably determined both by the material itself and the arrangement of that material. The distinctive tubule density and zonal tubule arrangement of the hoof wall of the horse is probably designed optimally in order to accommodate the functional demands of the hoof both at the level of the tubule and of the wall.

Considerable debate exists concerning the relative importance of the tubular architecture in determining the functional capacity of the hoof wall. For example, Leach (1980) suggested that, during weightbearing, ground reaction forces are transmitted proximally up the wall. Nickel (1938, 1939) and Wilkens (1964) believed the tubular and intertubular arrangement of hoof horn to be of importance in stress transfer and resilience, providing resistance to loading forces. The early work of Nickel (1938, 1939) considered that the tubules acted as vertical struts with the intertubular horn transferring stresses to the tubules. However, Thomason *et al.* (1992) reported no relationship between principal strain and the orientation of tubules and intertubular horn, although Chang *et al.* (1993) noted that the strain pattern was aligned with the major functional axes of the hoof wall tissue in horses and donkeys.

Reilly *et al.* (1996) suggested that the high tubule density in zone 1 may allow stress to be concentrated in the outer wall as a function of its load bearing properties and that the rapid decline in tubule density in zone 1, and the step like pattern of tubule density from zone 1 to zone 4, may be a mechanism for the smooth transfer of stress across the hoof wall to the axial skeleton. This mechanism of smooth energy transfer may work in conjunction with stiffness changes, mediated by changing hydration levels that have been reported by Leach (1980), Leach and Zoerb (1983) and Bertram and Gosline (1987). Reilly *et al.* (1996) concluded that the hoof wall is a multi-laminated composite structure. Transferring load as gradually as possible between interfaces is a guiding principle in composite technology and helps prevent failure. As well as transferring load, the presence of a large number of tubules per unit area in zone 1 may act to significantly increase the work of fracture and create a 'tortuous jagged path' to inward crack propagation. As energy is absorbed in the separation of 2 different phases (Gordon 1976; Vogel 1989) the tubular-intertubular interface may produce an effective crack stopping mechanism (Bertram

and Gosline 1986; Reilly *et al.* 1996; Kasapi and Gosline 1996, 1997). Distinct populations of tubules allow different crack diversion mechanisms to exist across the wall and would therefore safeguard against crack propagation originating in different directional planes. This has been confirmed by Kasapi and Gosline (1997) who reported 3 such distinct crack diversion mechanisms across the hoof wall.

The transition between 2 zones of differing tubular morphology and physical properties can result in reduced cohesive ability (Bolliger 1991). It can be argued that the areas of rapid tubule density change (zones 1 and 3) are liable to reduced cohesion. Zenker *et al.* (1995) reported that microcracks appeared most often at the transition between the inner and middle zones (i.e. in our zone 3). It is interesting to note the similarities in zone 3 tubule density values for horse and pony. This may represent an optimal value for this zone. In addition, Reilly *et al.* (1996) argued that other types of cracks seen in the hoof wall may be governed by the strength of the interfaces between zones. These may be mechanisms for the controlled elimination of damaged wall segments by zonal delamination of hoof wall layers along planes of designed inherent weakness. These would act as an effective means of preventing catastrophic failure (Bertram and Gosline 1986; Reilly *et al.* 1996; Kasapi and Gosline 1997). In this way, Reilly *et al.* (1996) suggested that the hoof wall may function as a quadrilaminar ply and Kasapi and Gosline (1997) a trilaminar ply.

Reilly (1995) emphasised that there is a need to develop an understanding of the anatomical and functional relationships between the various hoof horn parameters in normal equine hoof. In order to achieve this, these must be defined through objective measurements from recognisable anatomical sites. This study furthers the characterisation of one of these parameters, namely tubule density at the midline dead centre of the *stratum medium*. The results confirm a 4 zonal pattern similar to that reported for pony hoof by Reilly *et al.* (1996), and reinforces the suggestion that this is an equine pattern for construction of the hoof wall at this site. However, the differing tubule density values and zonal boundaries for horse and pony suggests a difference in finer detail between the two. It should also be noted that Hifney and Misk (1983) described tubular density differences between horse and donkey hoof. Reilly (1997) commented upon this, and presented photographic comparisons in support of these observations. However, these purported differences are still to be measured objectively.

Reilly *et al.* (1996) gave examples of the usefulness of a full understanding of hoof tubule density in research terms. Further work is required to establish tubule density values for equid populations and to test the precise mechanical consequences of this structural arrangement under controlled experimental conditions. It may then be possible to assess whether abnormal tubule density changes are associated with pathologically altered states, such as laminitis, and to evaluate the mechanical consequences of these changes.

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Manufacturers' addresses

[1]Adobe Photoshop, Adobe Systems Inc. California, USA.
[2]Minitab Minitab Inc. State College, Pennsylvania, USA.
[3]Microsoft Corp, USA.

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APPENDIX VI

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Finite element analysis of static loading in donkey hoof wall

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Keywords: horse; finite element analysis; donkey; hoof wall

Summary

A finite element model of donkey hoof wall was constructed from measurements taken directly from the hoof capsule of the left forefoot. The model was created with a 2 mm mesh and consisted of 11608 nodes. A linear elastic analysis was conducted assuming isotropic material properties in response to a 375 newton (N) load, to simulate static loading. The load was applied to the wall via 400 laminae in order to simulate the way in which the pedal bone is suspended within the donkey hoof capsule. Displacement, stress concentration, principal strain, and force distribution across the hoof wall were evaluated. The hoof wall model revealed loading responses that were in broad agreement with previously reported *in vivo* and modelled observations of the equid hoof. Finite element analysis offers the potential to model hoof wall function at the macroscopic and microscopic level. In this way, it could help to develop further our understanding of the functional relationship between the structural organisation and material properties of the hoof wall.

Introduction

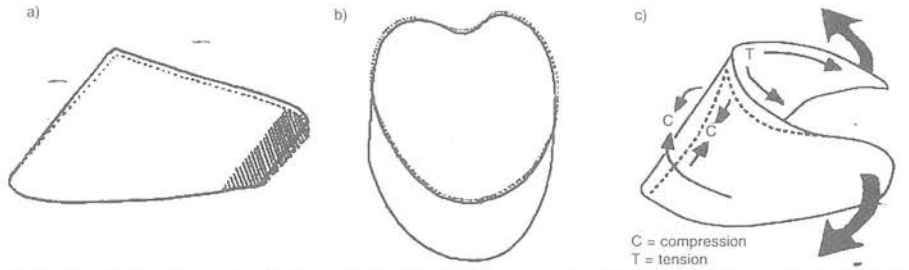
Leach (1980) suggested that the functional demands placed upon the equid musculoskeletal system are immense as, during both static and dynamic loading, the entire weight of the equid is directed through the foot and the hoof in particular. During locomotion the hoof strikes the ground with great force and frequency. For example, in the galloping horse a vertical concussive force of approximately 9000 N (Quddus *et al.* 1978), equivalent to twice the bodyweight of the animal, is generated during a stance of 0.1 s (Geary 1975), and at a frequency of up to 120 strides/min (Lekeux and Art 1994). In this respect, Dyhre-Poulson *et al.* (1994) noted that the anatomical structures of the foot are particularly adapted to absorb energy. However, the hoof capsule is fundamental to equid performance and has largely been overlooked in terms of biomechanical modelling. Modelling studies have tended to focus upon the role of the dermal structures and the bones during load bearing. However, the hoof must afford protection to the underlying sensitive structures of the foot. Hence it is essential that it is capable of withstanding the forces generated by ground impact (Douglas *et al.* 1996) and it must also dissipate the resultant shock waves to dampen concussive forces (Dyhre-Poulson *et al.* 1994). This must be achieved without excessive deformation or failure (Leach 1980; Bertram and Gosline 1986), as this would threaten the sensitive structures of the foot (Leach and Zoerb 1983). In this way, smooth and painless force transfer between the ground and the axial skeleton can be achieved (Reilly and Kempson 1992). As a result of these capabilities, Pollitt (1990) and Reilly *et al.* (1996) have described the hoof as a miracle of bioengineering.

Vogel (1988) stated that a biological preference exists for accepting rather than preventing deformation and Sack and Gabel (1977) suggested that the hoof capsule yields under the pressure of impact to dissipate concussive forces. Distortion of a solid object in response to an applied load generates internal forces sufficient to counter the applied load (Gordon 1976). Hence, during normal weight bearing and locomotion, the hoof deforms in a consistent pattern (Douglas *et al.* 1996) which results from a compromise between the complex force changes occurring internally within the capsule, and those external compressive forces acting against the ground (Leach 1980).

The hoof wall represents the major functional load-bearing component of the hoof capsule (Nickel 1938, 1939; Parker 1973; Sack and Gabel 1977). However, considerable scientific debate exists as to the means by which the hoof wall achieves its functional requirements. Rooney (1978) proposed that the structural design of the hoof reflects the need for force resistance and energy absorption. Therefore, an interpretation of hoof wall function necessitates an understanding not only of the structure and morphology of the wall, but also the complexities of the external and internal forces affecting the capsule, and the response of the wall to these forces (Leach 1980). There is, however, a distinct lack of scientific information regarding the nature and distribution of forces within the hoof wall during loading.

Studies dating back to the last century have attempted to evaluate hoof deformation in response to loading (Miles 1856; Lechner 1881; Bayer 1886; Förringer 1889). However, many of these earlier studies were significantly restricted by the technology available at that time. Nevertheless, Lungwitz (1883, 1891) was able to arrive at a model of hoof wall deformation that has, in the main, been substantiated by later workers including Fischerleitner (1974), Leach (1980), Colles (1989) and Thomason *et al.* (1992). Lungwitz (1883, 1891) recorded an inward movement of the anterior aspect of the hoof wall at the height of the coronary band that Leach (1980) referred to as a dorsoconcavity. In addition, Lungwitz (1891) recorded a concurrent expansion at the heels, flattening of the sole, and sinking of the heels which decreased the hoof wall height (Fig 1).

Bartel *et al.* (1978) proposed a model for the sagittal plane of the distal limb illustrating force transmission within the foot. A



(a) The unbroken line indicates the original shape of the hoof in the free loaded state. The dotted line illustrates the retraction of the coronary edge and the sinking of the heels. The shaded area indicates expansion at the heels. (b) The dotted line shows the geometric change in form of the hoof wall during load-bearing. (c) The unbroken line represents the shape of the unloaded hoof wall and the dotted line shows the change in form that occurs during loading.

Fig 1: Diagram to show the deformation of the hoof wall during loading, illustrating the expansion of the heels, the retraction of the leading edge of the hoof and the sinking of the heels (after Lungwitz 1891) and the force inter-action according to Leach (1980).

TABLE 1: Basic geometric measurements at the 10 hoof wall measurement sites in the lateral half of the hoof capsule

Site	y,z coordinates[a]	Tubule inclination (°degrees)[b]	Tubule length (mm)	Wall thickness at bearing border (mm)
1	[illegible]	40	57	10
2	10.2	40	58	10
3	20.6	40	57	10
4	30.15	40	56	10
5	35.28	40	56	10
6	36.38	40	54	10
7	36.48	40	52	10
8	39.58	40	48	10
9	39.68	40	43	10
10	38.78	40	40	10

[a]y,z Coordinates. y = distance in mm from the plane of the midline dead centre (MDC) at the bearing border (BB), along the y axis. z = distance in mm from the dorsal aspect of the midline dead centre (MDC) at the bearing border (BB), along the z axis.
[b]Tubule inclination from the axis in the x, z plane, expressed in °degrees.

vertically orientated ground reaction force was considered to act through the centre of the hoof capsule, counteracted by the downward force of the bodyweight acting through the distal phalanx. Components of this ground reaction force acted throughout the whole of the distal bearing border of the hoof wall, with a resolved force vector directed parallel to the hoof wall. An inwardly directed tensile force was suggested on the inner surface of the wall, orientated in an orthogonal direction. The former being resisted by the hoof wall itself while the latter was countered by the laminar junction. Leach (1980) suggested that the concussive wave is dampened as it travels vertically through the hoof wall, and that the internally directed tensile force is possibly increased by the action of the deep digital flexor tendon on the distal phalanx. The action of the flexor tendon would cause posterioventral rotation of the phalanx and a synchronous displacement of the hoof wall (Fischerleitner 1974; Leach 1980; Thomason *et al.* 1992) resulting in the observed dorsoconcavity.

Leach (1980) and Thomason *et al.* (1992) argued however, that such a model did not adequately take account of the 3 dimensional nature of the hoof capsule. They suggested that the compressive forces directed through the wall would result in tensile strain at right angles to these forces. Expansion of the heels in response to the posterioventral movement of the distal phalanx results in horizontally directed compressive forces at the toe - at right angles to those generated by the ground reaction forces. This would subject the wall to biaxial compression (Mair 1974). Furthermore, Geary (1975) observed that the extent of the laminar junction varies throughout the capsule resulting in tensile components being centred upon the anterior half of the hoof. Thomason *et al.* (1992) suggested that this results in the quarters being pulled inwards and downwards.

These studies have initiated debate regarding the precise nature of forces acting upon the hoof wall and have raised questions regarding its function (Nickel 1938, 1939; Rooney 1978; Leach 1980). Leach (1980) concluded that the hoof wall is likely to experience forces originating from 3 sources, namely compressive stress from the ground, tensile stress from the laminae and stress resulting from the change in form.

Various techniques have been employed to evaluate force distribution in the hoof wall. Strain gauges attached to the outer surface of the hoof have been used to monitor capsular deflections during loading (Mair 1974; Colles 1989; Thomason *et al.* 1992). However, it is important to appreciate the limitations of these studies - for example, sensors are restricted to monitoring deflections upon the outer surface of the capsule only. Also, the information provided is only site specific and may not be representative of capsule dynamics, and the location of the sampling sites themselves are often ill defined. In general, these studies have provided evidence that supported the proposals of Leach (1980), with compression predominating at all monitored sites. Strain patterns vary however between studies. Mair (1974) and Chang *et al.* (1993) detected biaxial compression at the dorsum, which is the anatomical site referred to as the midline dead centre (MDC) by Reilly *et al.* (1996), suggesting horizontal compression within the hoof. However, Thomason *et al.* (1992) recorded such strain patterns only in proximal MDC sites. Furthermore, strain patterns recorded at the quarters indicate that heel expansion does not occur in a simple horizontal manner (Thomason *et al.* 1992). Rather, the heel expands more at the distal margin than the proximal edge (Douglas 1994). This results in an outward and upward

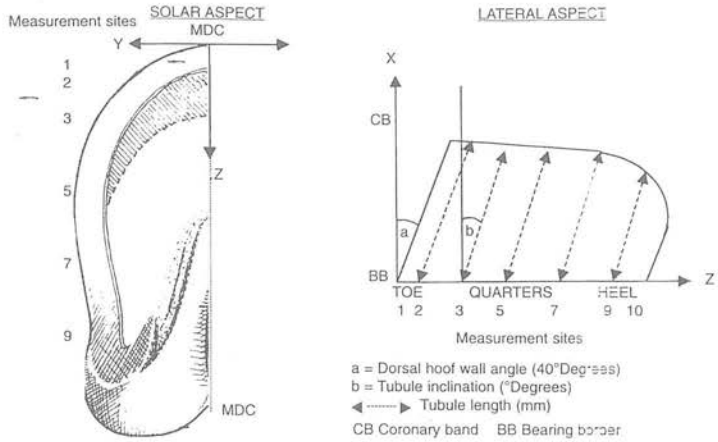


Fig 2: Diagrammatic representation of the basic geometric measurements used to construct the basal template and bounding curves of the donkey hoof wall Finite Element model.

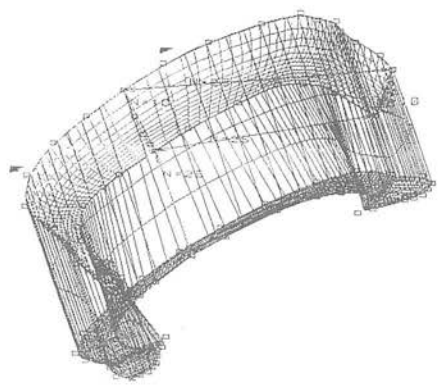


Fig 3: Surface model of the donkey hoof wall showing the initial mapping of the control points, splines and bounding curves.

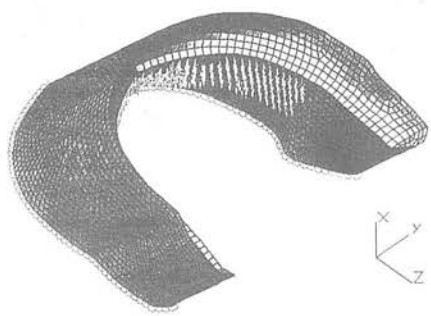


Fig 4: Finite element net construction model of hoof wall using a 2 mm mesh size, showing loading conditions (yellow arrows) and boundary restraints (red circles).

expansion of the hoof wall, generating compressive forces directed towards the coronary band at the MDC (Douglas 1994).

Davies (1997) and Dejardin *et al.* (1997) utilised photoelastic stress analysis techniques to document the distribution and magnitude of shear stress occurring on the outer surface of the hoof capsule in response to loading. This technique facilitates monitoring of the entire hoof capsule, therefore overcoming the 'site specific' limitation of conventional strain gauge analysis. However, this analysis is restricted to recording surface conditions only, and cannot objectively elucidate the nature of force distribution across the entire hoof wall depth.

Nickel (1938, 1939) assumed that the hoof wall was subjected to compressive forces alone, whereas Thomason *et al.* (1992) argued that the inner aspect of the hoof capsule was likely to be subjected to tensional forces, thereby intimating the occurrence of bending forces. In this respect, Hood *et al.* (1992) evaluated force generation in the hoof wall during loading using transducers capable of discriminating between bending and compressive deformation. They observed that the dorsal hoof wall (MDC) was subjected to either pure bending, or compression and bending. Pure compression alone within the wall, was not recorded.

Reilly (1995) argued that there is a need to further develop our knowledge of the mechanical functioning of the hoof wall as the mechanisms by which the hoof wall successfully achieves its function are not fully understood. The relationship between the forces generated during loading and the structural organisation and biomechanical and material properties of the hoof need to be established. Computer assisted 3 dimensional modelling of the hoof wall using finite element analysis (FEA) may serve as a means of elucidating the complex interaction of forces generated during loading, and provide essential information that is required to unravel the functional complexity of the hoof wall.

Several authors have used this mathematically based modelling technique to investigate internal deformations and stress distribution in different biological structures including bone and tendon. However, its application to the hoof has been limited. Wichtmann *et al.* (1990) and, Hood *et al.* (1991) used this technique to produce a 2 dimensional model of the equine foot in the sagittal plane in order to evaluate the theoretical

force distribution within the various structures of the digit in response to static loading. Hinterhofer *et al.* (1997) employed FEA to investigate the effect of dynamic loading upon a 3 dimensional model of the hoof capsule. This paper represents a logical progression to these former FEA studies and marks the first in depth 3 dimensional simulation of the major load bearing element of the hoof capsule, namely the hoof wall during static loading.

Materials and methods

Application of finite element analysis to the hoof wall

The Finite Element Analysis (FEA) method is widely used in the engineering industry and associated fields, and can be applied to static, dynamic, thermal, electrostatic and fluid flow problems. Used in static analysis it essentially relies upon developing mathematical equations that relate the displacements created, to the forces applied to small discrete subregions of the structure or 'Finite Elements'. These Finite Elements can be of various geometrical configurations i.e. rod, brick, or plate-shaped. The ends or corners of the element are called nodes. When these elements are assembled to represent the structure, the occurrence of common nodes, i.e. those which are common to adjoining finite elements, allow mathematical equations to be assembled to determine how the structure reacts theoretically to the applied forces. The derived displacements can then be used to determine strains and stresses. In order to restrain the structure and prevent free body movement, boundary conditions need to be applied. These have to be carefully considered in order to recreate accurate *in vivo* conditions.

The application of Finite Element Analysis to the structural performance of the hoof wall extends and complements the range of experimental techniques that have formerly been used to evaluate hoof wall function. Confidence in such an analytical technique should always be developed by comparison with experimental work, although care must be taken that like is compared with like. For example, the application of photoelastic material to the surface of a hoof in order to assess stress levels with unavoidably reinforce and stiffen the structure that is being assessed and, if bending is the major stress developing process, this may critically alter results. The aims of this study were to design a finite element model of the hoof wall of a donkey, and to model the effect of static loading upon the structure. This assumed linear elastic stress - strain relationships and isotropic material properties within the hoof wall. The study also aimed to evaluate the potential application of finite element techniques to hoof wall mechanics by comparison of simulated loading responses with previously reported *in vivo* observations and modelled data in the equid hoof capsule.

Developing the model

The model was based upon measurements of a hoof capsule obtained from the left forefoot of a donkey, which had no apparent signs of disorders related to the feet, that had been humanely destroyed on medical grounds. Immediately after destruction, the hoof capsule was sealed in Parafilm to prevent shrinkage resulting from moisture loss, and was stored in a fridge until autolytic degradation of the dermal/epidermal junction allowed removal of the intact hoof capsule. Measurements were taken immediately after capsular removal to minimise the effect of moisture loss.

In order to create a successful model of a shape, such as the hoof wall that is composed of many intersecting curved surfaces, the use of a surface modeller is required. In this case, the initial shape of the bounding curves was developed. To this end the solear aspect (bearing border) of the hoof wall was accurately measured and mapped into the computer. In this way a basal template was constructed to which all subsequent measurements could be related spatially (Fig 2). The position of the coronary band was established by measuring the heights along the longitudinal axis of the horn tubules, from the bearing border to the coronary band, at ten sites from the MDC to the heels in the lateral half of the capsule (Table 1). The angle of inclination from the x axis was measured in the x, z plane. In this way, a series of control points were created and splines, a form of mathematical curve, fitted to the control points to form the bounding curves. These splines were transferred to the surface modeller and with the addition of some connecting lines, the bounding areas were created. Once this had been completed for one half of the hoof wall model it was mirrored about the plane of the MDC to form the complete hoof wall structure. These were then patched to create the surfaces of the model (Fig 3). These were subsequently meshed to form a finite element net construction on the surfaces of the wall model. Specific attention was given to ensure that the mesh size was small enough to allow the development of modelling detail, yet large enough to avoid an excessive computer file size.

This surface mesh was then enhanced to generate a more regular model, and brick elements generated to fill the volume using a finite element software package[2]. Once more, mesh size selection was critical, as not only does the mesh size affect the accuracy and detail of the final results, it also governs the processing time required for the analysis. This ultimately determines the feasibility of the analysis. For example, although a 1 mm mesh size provides a model capable of generating exceptional detail, the mesh enhancement process alone would require 16 h computer processing time on a Pentium 75 mHz PC to create ~45,000 elements. Therefore a 2 mm mesh size was selected. This required 4 h to generate and gave a respectable model with adequate detail, consisting of 18305 elements and 11608 nodes. A general arrangement, light shaded view, of the 2 mm mesh models is shown in Figure 4, with arrows indicating the loading surfaces and red circles indicating the boundary restraints.

A vertical static load of 375 N was used for the model. This represents a static loading force equivalent to a 150 kg bodyweight animal distributed equally between the 4 hooves. One hundred and fifty kg is a typical bodyweight for a donkey (Chang *et al.* 1993). This force was applied uniformly around the wall in a manner designed to simulate the suspension of the axial skeleton within the hoof capsule (Sack and Gabel 1987), via 400 laminae (Hifney and Misk 1983). The bearing border of the hoof wall was restrained vertically to simulate *in vivo* ground contact conditions. In addition the bearing border at the MDC was fully restrained preventing displacement at this anatomical site in any direction. These boundary conditions were consistent with the observations of Lungwitz (1883, 1891) and video footage published by Pollitt (1993) for the horse. The hoof wall was assumed to be isotropic with a Young's Modulus of 0.5×10^3 N/mm^2 (Wichtmann *et al.* 1990) and a Poisson's ratio of 0.4 (Chang *et al.* 1993). The analysis required the solution to 34,261 equations which took approximately 2 h computer processing time on a Pentium 75 mHz PC.

Results

Figure 5 illustrates the deformation of the hoof wall model in response to loading and boundary restraints. A comparison of the displaced shape with the original unloaded structure revealed an outward displacement at the heels, which progressively increased towards the palmar aspect attaining a maximum value of 2.36 mm. A dorso-concave deformation of the dorsal aspect of the MDC was indicated with the proximal region experiencing an inward deflection of 0.3 mm. The 'total deflection' at any point around the hoof wall model in the loaded state is shown in Figure 6. The maximum principal strain in the outer aspect varied around the hoof wall. In general strain values increased from the heels towards the MDC, attaining a maximum value in the order of 2500 με at the proximal region of the MDC (Fig 7). The direction of the principal strains indicates that the maximum principal strain at the MDC was aligned along the x axis both proximally and distally, and that the hoof wall at this site was subjected to biaxial compression. The biaxial compression was greater proximally than distally.

A modelled sagittal section taken along the plane of the MDC (Fig 8) revealed that the dorsal aspect of the hoof wall was subjected to compressive forces which increased progressively in a proximal direction from the bearing border. These compressive forces decreased across the hoof wall, in the dorso-palmar direction, with the development of tensile forces towards the inner margin of the wall section. The greatest tensile force was generated in the proximal half of the wall.

Discussion

The displacement diagram and displaced shape projections reported in this paper predicts a pattern of donkey hoof wall deformation, in response to static loading, which is in broad agreement with *in vivo* observations for the horse as reported by Lungwitz (1889, 1891) and Pollitt (1993). The simulated loading, resulting in an outward expansion of the heels and a simultaneous dorsoconcavity of the proximal MDC suggests that the mode of mechanical functioning of the donkey hoof wall may be similar to that of the horse. The displacement diagram illustrates that, in the region of the quarters and heel, the displacement value is greater proximally than distally. This contradicts *in vivo* observations in the horse where lateral expansion is greatest at the bearing border reported by Thomason *et al.* (1992). However, it is important to note that the displacement values are nominal, that is they give absolute displacement values only, and are not vector quantities giving directional information. The displaced space plot indicates a uniform outward expansion. Therefore, the predicted values suggest that the proximal region of the quarters may be subjected to a combination of different compressive, tensile, bending and torsion forces. Thomason *et al.* (1992) suggested that the proximal region of the heels in the horse were subjected to an inwardly directed tensile force that results in an outward and upward movement of the heel distally. However, the displacement plot in the y direction for the donkey (Fig 9) predicts uniform outward expansion proximally and distally and, therefore, it suggests that axial compressive forces may be contributing to the displacement at this site. This may reflect the sophistication of the model and the assumptions made in this respect, or may represent a genuine difference between horse and donkey. Reilly (1997) commented upon the anatomical differences between horse and donkey hoof capsule, and suggested that there may be different amounts of movements within the 2 capsules during loading. It can be argued in terms of mechanical principles, that geometrical differences are likely to affect displacement under loading. In this case, heel expansion is likely to be less in the more upright donkey capsule due to increased axial resistance. In addition, tapering of the hoof wall depth in the heel region of the horse (Reilly 1997) presents less material to resist displacement, and increases the tendency to accommodate such movement.

The theoretical distribution of principal strain around the outer surface of the hoof wall indicates the occurrence of maximum principal strain centred upon the proximal region of the MDC. This is consistent with experimental observations using surface strain gauges for the horse (Thomason *et al.* 1992; Chang *et al.* 1993; Kasapi and Gosline 1996) and for the donkey (Chang *et al.* 1993). This suggests that this FEA model is reproducing *in vivo* conditions. In addition, the principal strain distribution in the hoof wall model was comparable with that reported in the donkey hoof by Chang *et al.* (1993).

The stress distribution at the surface of the capsule that is derived from the strains using the stated Young's Modulus and Poisson's ratio can be displayed in various forms. The magnitude of the Maximum Principal stress can be plotted as can the Minimum Principal stress, or, a combination of these related to various failure theories. However, given the complexity of the structural hierarchy evident in the hoof wall (Reilly *et al.* 1996; Kasapi and Gosline 1997), and our limited knowledge of the material properties of hoof horn (Reilly 1995), it is difficult to say which failure mode is applicable. However, 2 stress states may be of interest, (i) The Maximum shear stress (Tresca) and (ii) The Von Mises Stress (Shear strain energy theory). Both of these are usually related, in engineering applications, to failure in ductile materials. Such a mode of failure may be applicable to hoof wall modelling due to the potential plasticising effect of the material's inherent moisture content (Cope *et al.* 1998). However, as the material is non isotropic and possibly behaves in a nonlinear fashion when loaded at high speed, these values can only give an indication of the resultant conditions. The accuracy of the results can be as accurate only as the assumptions made in developing and resolving the model. The Shear stress plot is also interesting in that it relates to the isochromatic fringes produced in photoelastic analysis. A plot of 2x the Tresca stress is used to simulate photoelastic surface strain and is used as a comparison (Fig 10).

The accuracy of FEA is dependent upon contributions from the geometrical modelling of the structure, the selected mesh size, the specified material properties, the defined boundary conditions, and the chosen load and means of loading. Whilst confidence can be expressed in terms of the geometric modelling and the boundary restraints, several important assumptions and compromises have had to be made with respect to the other factors.

The accuracy of the solution is related to the mesh size. The software used in these simulations can provide an indication of accuracy by calculating a precision value. It takes the values of the quantity of interest, for example stress, and compares the values derived at a node from all the elements connected at that node. These will differ depending on the mesh size and the local stress/strain gradients. The precision is defined as the maximum difference between these values divided by the maximum stress in the body. In this way it can be seen where the mesh needs to be refined to improve future accuracy.

Accurately inputting material properties into the model represents more of a challenge because there is a distinct lack of

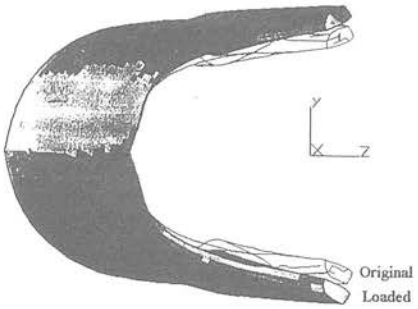


Fig 5: Displaced shape diagram of modelled hoof wall deformation in response to loading, illustrating the deformed/displaced wall section compared to the original unloaded net construction model.

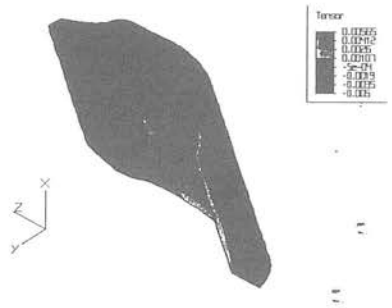


Fig 8: Force distribution along the sagittal plane in the region of the midline dead centre (MDC) during static loading.

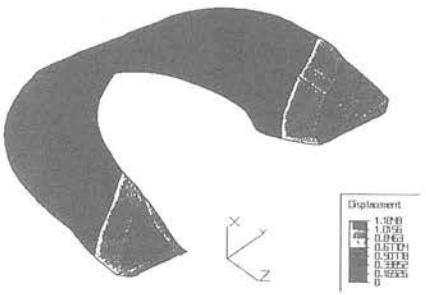


Fig 6: Displacement plot of total displacement (mm) at all points around the loaded hoof wall model.

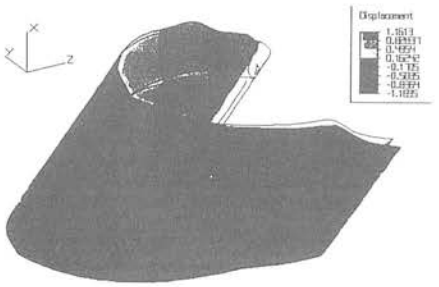


Fig 9: Projection of hoof wall displacement along the y axis in response to loading onto the original net construction.

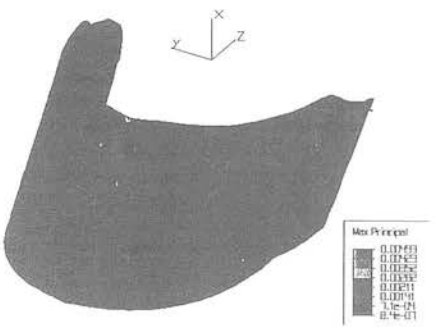


Fig 7: Shaded plot representation of Maximum Principal Strains generated in the hoof wall model in response to loading.

Fig 10: Plot of 2x tresca stress during loading to simulate photoelastic surface strain analysis.

information regarding the material characteristics of donkey horn. Therefore a representative equine modulus of elasticity and Poisson's ratio (Wichtmann *et al.* 1990) was selected for this FEA investigation. Anecdotal evidence suggests that donkey horn is not as stiff as that from the horse. Since, the modulus of elasticity affects the resultant principal strain the higher the modulus, the lower the principal maximum strain. On this basis, the use of an equine modulus is likely to have resulted in lower strain levels than occur *in vivo* for the donkey. Experimental work is currently in progress to characterise the material properties of donkey hoof in order to enable refinement of this model.

Although the force generated by an average donkey bodyweight during static loading can be calculated with relative ease, there is a lack of scientific evidence as to how this force is distributed between the 4 hoof capsules. Kainer (1987) stated that the forefeet support 60% of the bodyweight of the horse. If a similar situation exists in the donkey then the results of this study, based upon an even force distribution acting through each hoof, under-represents the effect of static loading upon the deformation and stress distribution in the forelimb donkey hoof wall. In addition, there is limited information regarding the means by which the force is transferred from the axial skeleton to the ground via the hoof capsule. It is not known if the load is transferred equally via the 400 laminae or whether more weight is taken at the MDC as suggested by Douglas *et al.* (1996) in the horse. Foot balance has also been reported to affect the loading characteristics of the hoof (Chang *et al.* 1993). This study assumes mediolateral foot balance, although there is limited information regarding natural foot balance in the donkey. Williams (1993) suggested that the donkey naturally takes more weight on the medial aspect of the bearing border in the hindfoot, however it is not known whether the same is true for the forefoot. This study is also based upon the assertion that the hoof wall constitutes the load-bearing element of the equine hoof capsule (Nickel 1938, 1939), but there is debate as to whether the sole in the donkey should be load-bearing (Reilly 1997). For example, Whitehead *et al.* (1991) and Fowler (1995) stated that the anterior third of the sole plays an important role in load-bearing. Reilly (1997) referred to the present lack of knowledge concerning the extent to which the sole bears load or whether in fact, the sole naturally should be concave and hence bear no load.

The application of FEA to hoof mechanics has been limited. The different modelling techniques employed, and the working assumptions made, make direct comparison difficult. Wichtmann *et al.* (1990) and, Hood and Wichtmann (1991) modelled a 2 dimensional sagittal section at the MDC of the equine foot, loaded via the second phalanx. This model of the foot consisted of only 964 nodes and 457 elements and therefore did not afford modelling detail. Compression bending and rotation of the wall were described resulting in dorsoconcavity of the proximo dorsal aspect of the hoof wall, consistent with the results of this study. However, such a 2 dimensional representation cannot model the effect of compressive forces generated at the MDC by heel expansion, nor the tensile forces generated orthogonal to the tubular axis as described by Leach (1980) and Thomason *et al.* (1992). Consistency of dorsoconcavity between this present study and Wichtmann *et al.* (1990) supports our assumption that it is possible to model the hoof wall in isolation and still generate accurate *in vivo* simulation. Hinterhofer *et al.* (1997) working with a more sophisticated model in terms of the material properties of the capsular components, were not able to create *in vivo* deformation within the wall, unless part of the load was applied via the sole/frog. However it is not clear how the white line, which forms the wall sole junction was modelled. It is believed that the white line allows for independent movement of the wall and sole (Reilly 1997). Comparison of displaced shape and stress concentration plots reveal similarity between this study and Hinterhofer *et al.* (1997). However it is uncertain whether a hoof capsule subjected to forces of ~30000 N, as modelled by Hinterhofer *et al.* (1997) would function in a linear elastic manner. Further work is in progress to validate whether modelling the hoof wall in isolation accurately simulates *in vivo* conditions.

There are many advantages in applying FEA to this type of mechanical problem. Firstly, it is possible to generate a general understanding of the structure and analyse how it performs under various loading conditions. In particular it is possible to determine with reasonable confidence, conditions in regions of the hoof wall where experimental results are difficult to achieve. Secondly, it is possible to work on a micro as well as a macro level so that a more detailed analysis of the structural complexity of the hoof wall, and its performance, can be achieved. Finally, FEA provides an ideal focus for bringing together the information derived from a multidisciplinary approach to investigating hoof function, as advocated by Reilly (1995).

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APPENDIX VII

EQUID HOOF HORN: A NATURAL COMPOSITE

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ABSTRACT

A finite element model for the stratum medium of the equid hoof was developed at the microscopic level of its structural hierarchy based upon a morphometric analysis of the 'outer' region of the hoof wall at the midline dead centre of the donkey hoof capsule. A 2-dimensional plain strain finite element analysis (FEA) was conducted assuming isotropic material properties to generate mean modulus values in the lateral directions over a range of tubule to matrix stiffness ratios. Axial modulus values were also obtained based upon the 'rule of mixtures'. Solutions obtained from the FEA closely correspond with those obtained by the modified Hull equation, and, at a tubule to matrix modulus ratio of ~3.5- 4.0, the axial to lateral modulus ratios were in broad agreement with experimental data reported for the hoof. These results lend support to the assertion that the stratum medium may act as a unidirectional fibre composite at this level of the structural hierarchy, with the horn tubules acting as a fibre phase dispersed within a matrix of intertubular horn. The tubule and matrix strain concentration may be of significance with regard to energy absorption and crack propagation.

INTRODUCTION

The equid hoof is a natural biological composite composed of keratinised material Cope et al (1) that serves to suspend the skeleton and protect the sensitive structures of the foot. It must therefore be capable of accommodating and resisting high loads (up to 600 kg) on uneven surfaces and at high speeds without excessive deformation or catastrophic failure Leach (2). Consequently it is essential that it is capable of withstanding the forces generated by ground impact Douglas et al (3) and it must also have the ability to dissipate the resultant shock waves in order to dampen the concussive forces Dyhre Poulsen et al (4). Vogel (5) stated that a biological preference exists for accepting rather than preventing deformation. Hence during normal weight bearing and locomotion, the hoof deforms in a consistent pattern Douglas et al (3) thereby generating internal forces sufficient to counter the applied load. In this way, smooth and painless force transfer between the ground and the axial skeleton can be achieved Reilly et al (6). With respect to these capabilities, Pollitt (7) and Reilly et al (8) have described the hoof as a miracle of bioengineering.

However there is considerable scientific debate concerning the precise means by which the hoof wall achieves its functional requirements. Reilly et al (6) argued that a compromise must exist between the need to accommodate forces, minimise crack propagation and avoid excessive deformation that would threaten capsular integrity. Rooney (9) proposed that the structural design of the hoof reflects the need for force resistance and energy absorption. Thus, an interpretation of hoof function necessitates an understanding of the structure and morphology of the hoof, the complexities of the external and internal forces affecting the

capsule, and its response to these forces Leach (2).

Vogel (5) stated that the biomechanical properties of a structure are likely to be determined both by the material itself and the arrangement of that material. In addition Reilly et al (6) argued that the hoof is likely to be optimally designed in order to meet the functional demands placed upon it. In this respect, the equine hoof displays a complex hierarchical structural organisation evident at the gross anatomical, macroscopic, microscopic, ultrastructural and molecular level. Reilly et al (8) stated that the finer functional properties of the hoof are likely to be dictated by contributions from the structural organisation operating at many levels within the hierarchy, and these may be modulated by moisture content.

At the gross anatomical level the hoof capsule can be divided topographically into three main regions namely the wall, sole and frog. The hoof wall represents the major functional load-bearing component of the hoof capsule Nickel (10), Sack and Habel (11). Of the three morphologically distinct layers that form the hoof wall at the macroscopic level of the structural hierarchy, the stratum medium is considered to be the principal component responsible for determining its load bearing capabilities Pollitt (7), Kasapi and Gosline (12). At the microscopic level the stratum medium exhibits a distinct structural architecture consisting of a parallel array of keratinised horn tubules dispersed in a keratinised cellular matrix of intertubular horn Nickel (10), Wilkens (13). The horn tubules are comprised of a central void or medullary cavity, surrounded by a cellular keratinised cortex and extend from the coronary band to the weight-bearing border. In this way, at this level of the structural hierarchy the stratum medium has been considered as a unidirectional fibrous composite Reilly et al (8) with the tubules acting as the reinforcing material of the continuous phase Cope et al (1).

The relative importance of the tubular architecture in determining the functional capabilities of the hoof wall is unknown. However, if the hoof wall functions as a fibre composite then composite theory dictates that the mechanical properties of the structure will be determined by the material properties of the respective components, their volume fraction, and the orientation, size and shape of the reinforcing fibres.

Estimates of the mechanical properties of the hoof wall have been directly attained from a number of studies using compressive, tensile and bend test techniques Leach (2), Douglas (3), Kasapi and Gosline (12). However these tests have provided a wide range of values. These differences may reflect upon differences in testing protocol, moisture content of the material, sampling sites and inherent variability between individual animals.

Reilly et al (14) explained the need for careful control in experimentation and also emphasised the need to develop an understanding of the anatomical and functional relationships between the various hoof horn parameters in normal equine hoof. In order to achieve this, these must be defined by objective measurements from recognised anatomical sites. In addition, as there is a distinct lack of scientific information regarding the nature and distribution of forces within the hoof wall during loading, there is also a need to further develop our knowledge of the mechanical functioning and material properties of the hoof

wall.

Various theoretical modelling techniques have been successfully applied to synthetic composites in order to predict mechanical performance both at a macroscopic and microscopic level. Traditional macromechanical techniques, such as 'simple rule of mixtures', deal only with the overall response of the material to an applied load and deal specifically with the resultant gross stiffness of the structure. On the other hand more advanced techniques, such as the finite element approach, can also evaluate the specific interactions of the respective components at the microscopic level. In this way a micromechanical approach can provide a means of evaluating the effect of local geometric and morphometric properties such as tubule distribution, tubule shape and size, and porosity effects arising from the medullary cavity. In this respect Hogan (15) argued that modelling and analysis techniques which incorporate the distinctive microstructural components should provide a more accurate and deterministic understanding of gross mechanics and function. In this way, computer assisted modelling of the hoof wall using finite element analysis (FEA) may serve as a means of further elucidating the complex interaction of forces generated during loading, and provide essential information that is required to unravel the functional complexity of the hoof wall.

A finite element model of the donkey hoof wall has already been used to develop an understanding of the stress and strain distribution generated within the structure during static loading Newlyn et al (16), and the resultant deformation. Modelling at the gross anatomical level of the structural hierarchy has predicted results that are in broad agreement with *'in vivo'* observations of capsular deformation and surface strain values given in the literature Thomason et al (17).

This study aims to build upon our former work by objectively measuring the morphometric properties of the donkey hoof wall at the microscopic level and to use this information to model the hoof wall at microscopic level of the structural hierarchy. In this way, the relationship between structural organisation and mechanical behaviour of equid hoof horn can be investigated and the assertion that the hoof wall acts as a unidirectional fibre composite tested.

MATERIALS AND METHODS

Morphometric determination of the hoof wall.

Histological sections were prepared from hoof wall samples taken from the midline dead centre (MDC) sampling site of the left forefoot of 6 donkeys, according to Reilly et al (8). Digitised images of each section were captured using a microscope-mounted video camera. Initial image acquisition was performed with a 4 x objective and a 3.3 x photo-eyepiece. This combined with a 1.25 x gain arising from the configuration of the trinocular microscope head, resulted in a total magnification of ~ 20 x. The captured images were imported into a computer based image analysis system and subsequently analysed using the NIH-Image software program. A three stage semi-automated method has been developed that provides both dimensional and area measurements, and area fraction data. Measurement criteria were selected to include length of the major and minor axes, and area measurements. By this means absolute dimensional measurements of marrow, cortex and tubule, and area fraction information for the tubular and intertubular components were determined. Measurements

were conducted at 5% hoof wall depth of the stratum medium (HWD). This site was considered to represent the 'outer' hoof wall region referred to by Douglas et al (3) and Kasapi and Gosline (12)

Development of the FEA microstructural model

The morphometric analysis provided the data to construct a finite element model (see Figure 1) of the structural and geometric arrangement of the hoof wall. In developing this model, the microstructure of the hoof wall was considered as a 2 component (phase) hollow fibre reinforced composite. Each phase was treated as an isotropic linearly elastic component with perfect bonding assumed at the interface between the horn tubules and the intertubular horn. The geometric structure was simplified by assuming a hexagonal array of repeating horn tubule units each possessing an elliptical cross section in line with the mean major to minor axis ratio. The absolute dimensions of the horn tubule, cortex and medullary cavity were similarly based on the mean recorded values. The inter-fibre spacing combined with the absolute tubule dimensions, was used to generate a fibre density (tubule density) consistent with the area fraction data recorded

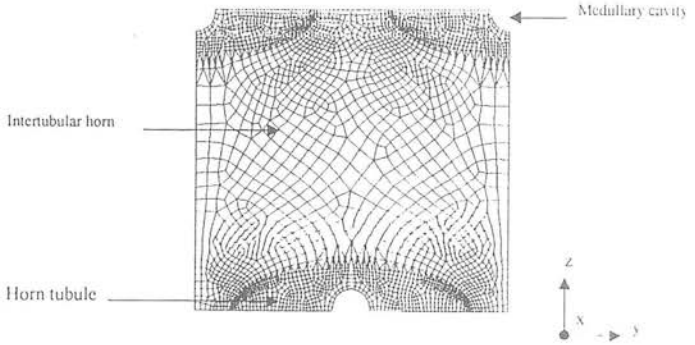

Figure 1 – A simplified 2-phase finite element microstructural model of a transverse section of the 'outer' hoof wall region to show the structural and geometric organisation of the horn tubules and the intertubular horn

Determination of the possible Moduli.

The Youngs modulus (E) for the composite can be approximated using the simplified equations for unidirectional laminae as explained in Hull (18). There are difficulties in applying these equations directly to equine hoof, as neither the modulus of the tubule nor the intertubular matrix modulus is known. However, if a modulus ratio (K) of E tubule to E matrix (Et/Em) is introduced then an assessment of the possible variation in moduli for a range of E ratios is possible. This can then be used to investigate experimentally determined values of modulus with a view to extracting a tubule and matrix modulus.

Table 1 – Axial and lateral modulus values reported for the equine hoof

Source	Hoof Wall Region	Axial modulus (Ex) N/mm^2	Lateral modulus (Ey) N/mm^2	Axial to lateral moduli ratio	Moisture content
Douglas et al (1996)	Outer	998	912	1 : 1.09	27.9%
Kasapi and Gosline (1997)	Outer	560	310	1 : 1.81	35.0%

A range of values for the Modulus ratio K, (Et/Em) was selected based upon previously reported mechanical testing data as shown in Table 1. From this information it can be seen however, that the ratio of axial modulus (Ex) to lateral moduli (Ey) is not well defined. Figure II shows that if equations for axial and lateral modulus are used, at a tubular volume fraction of 0.2 (mean value for donkey hoof wall - Collins unpublished data), then K values between 0.28 – 3.50 provide data that lies within the range of experimental results reported for equid hoof horn

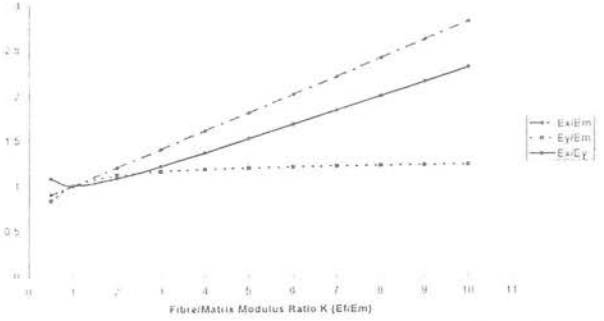

Figure II – Variation in the theoretical modulus ratios in the x and y direction with increasing tubule/matrix modulus ratio as determined by 'rule of mixtures'.

Based upon these values and the area fraction data obtained from the morphometric analysis, the axial and lateral moduli were determined using 'rule of mixtures' and the modified Hull equations. In this way, theoretical moduli were determined for the 'outer' hoof wall region

It was assumed that the volume fractions of tubular and intertubular horn were the same as the area fraction values determined experimentally. In addition by expressing the predicted moduli in Ex and Ey directions by convention as a ratio to the matrix modulus Em, the reinforcing effect of the structural organisation upon the matrix material can be evaluated for different values of K. By this means, it can be anticipated that a fibre composite structure reinforced in a given directional plane would display an E modulus in that direction > Em.

FEA Macromechanics

FEA was used to determine the lateral (Ey) modulus by a fixed displacement approach. This was achieved in each direction by applying a unit displacement on one edge in the direction required, and restraining the other edge with stiff boundary elements of known stiffness constant

and allowing poisson contraction. The mid-point of the restrained edge was fully restrained such that no displacement was possible in any directional plane. All other nodes were free to move. Plain strain 2-dimensional elements were used. The modulus was then determined by assessing the force in the boundary elements and the overall displacement in the chosen direction. This analysis was performed over a range of ratios of Et/Em from ~0.28 to 3.5. The FEA results derived from the analysis and the calculated values from the modified Hull equations have a restriction placed on strain in the x direction, and at a fibre to matrix modulus ratio of unity, indicate an axial to lateral modulus ratio >1. As the results are to be compared with modulus ratios obtained from tensile, compression and bend tests, where there is no strain restriction, the results were factored to pass through unity at a fibre to matrix ratio of 1.

FEA Micromechanics

Further interpretation of the FEA was conducted to investigate the micro stress/strain concentration developed as a consequence of the microstructural organisation. In this way, the effects of tubular shape upon the mechanical properties of the hoof could be further elucidated. Whilst it is appreciated that this approach cannot be used directly to assess possible failure, given that the tubular and intertubular components are likely to possess differing material properties, it may indicate areas of potential weakness. Such weaknesses may be of significance in crack initiation and stopping phenomena.

RESULTS

Morphometry results

The results of the morphometric analysis are summarised in Table II. The 'outer' hoof wall region was characterised by the presence of small, highly elliptical tubules, whose major axes were aligned parallel to the y or lateral axis. The tubular area fraction within this 'outer' region was 17%.

Table II – Morphometric analysis of the 'outer' hoof wall region

Hoof Wall Region	Percentage hoof wall depth (%HWD)	Tubule area fraction	Minor to major axes ratio	Mean tubule area measurement μm^2	Calculated tubule density Tubules mm^2
Outer	5	0.17	1 : 3.07	6899	24.9

FEA Results

Figure III shows modulus ratios for the 5%HWD model for a range of ratios of Et/Em from 0.2857 to 3.5. The axial modulus (Ex), was derived from the 'rule of mixtures' relationship. It can be seen that the ratio of lateral to axial moduli Ey/Ex reduces consistently as the reinforcing in the axial direction becomes more dominant. At an Et/Em ratio of 3.5 the Ey/Ex ratio is 0.9. This indicates that there is a relative stiffness of this order between the keratinous material in the two respective phases, and that it is the structural organisation of this material alone, that accounts for the difference in moduli. At this K value the axial modulus is ~39% stiffer than the matrix material.

The stress strain concentration factors resulting from loading in the y direction are given in Figures IV. Analysis of this data revealed that in the matrix, the maximum stress and strain

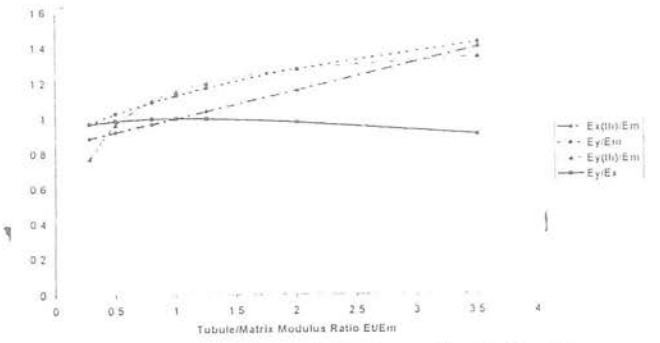

Figure III – Comparison of modulus ratios for the 'outer' hoof wall model.
Ey - derived from FEA. Ey(th) – derived from the modified Hull equation. Ex - derived from 'rule of mixtures'. Em - matrix modulus.

concentration factors did not exceed twice the mean values at the maximum Et/Em ratio of 3.5. However within the tubular component, the maximum strain is up to three times the mean at Et/Em of 1 and progressively reduces as the tubule modulus increases. By extrapolating the results it would seem that at Et/Em of ~ 4 - 4.5 the ratio of maximum to mean strain value would be the same in the tubule as in the matrix at ~ 2.25.

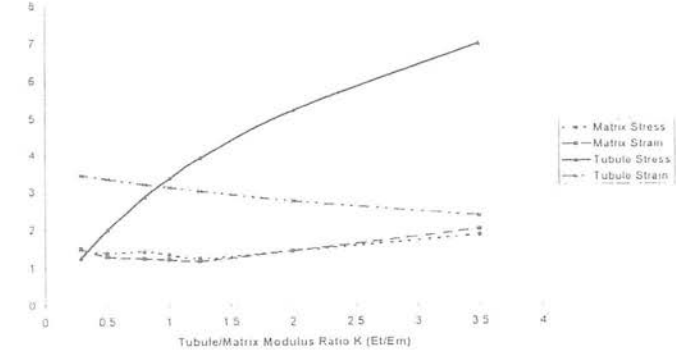

Figure IV – Variation in the stress/ strain concentration factors in the tubule and matrix components of the 'outer' hoof wall model with increasing tubule/matrix modulus ratio.

The distribution of the regions of maximum strain within the matrix was dependent upon the K value. When loaded in the y direction, the point of maximum strain moved as the relative stiffness of the tubule increased from unity, from a position at the tubular-intertubular interface

adjacent to the apex of the tubule minor axis to the apex of the major axis. With regards to the strain distribution within the tubular elements, the position of maximum strain was located at the boundary of the medullary cavity normal to the direction of loading.

DISCUSSION

This paper represents a logical progression to our former FEA modelling studies conducted at the gross anatomical level Newlyn et al (16). It represents the first study to use this technique to investigate the macromechanical and micromechanical behaviour of the equine hoof wall, during loading, at the microscopic level of the structural hierarchy. The information provided enables further refinement of our hoof wall model and will help unravel the function / design complexity of the equine hoof capsule.

Confidence in modelling techniques should always be tested by comparing the output with the results from experimental work. In this respect the results of FEA combined with simplified theoretical composite equations are consistent with the experimental data from mechanical tests reported by Douglas et al (3). Such an agreement, in itself, does not confirm that hoof horn is truly a composite material but the findings reported here do lend further support to the assertion of Reilly et al (8) and Cope et al (1) that it may operate as a unidirectional fibre composite.

Whilst the value of the ratio of axial to lateral modulus at < 2 may appear to be very small in comparison with those regularly encountered in synthetic composites it is not unexpected as both the tubular and intertubular horn are essentially different structural arrangements of the same cellular material. This is typical of many biocomposites. Consequently the ratios of Ef to Em are typically low in comparison with synthetic composites. Although no previously published values exist for equid hoof horn Hogan (15) cites Ef/Em estimates for cortical bone ranging from 1:1 to 10:1.

Similarly, the tubule volume fraction values may be considered to be relatively low when compared to synthetic composites. However, Saliba (19) stated that natural composites are optimum structures best suited to an organism in its natural environment. In this respect, the hoof wall may be optimised naturally to achieve a balance between accommodation and resistance of loading forces so as to dampen concussive forces without failure. It is also important to note that material in the MDC of the hoof wall is subjected to biaxial compression during static loading Thomason et al (17), Newlyn et al (16). In this respect a material that exhibits a modest Ex/Ey may be adaptive. In addition, Curiskis and Feughelam (20) modelling composite fibres reported that the greatest rate of change in Ey/Ex for a given fibre volume fraction increase, occurred in the range of 0-25%. Thus, the volume fraction of the hoof wall may be optimised in order to achieve the greatest potential modulation of the mechanical properties at least cost to the animal.

The results of this study demonstrate that the use of FEA with models derived from the morphometric analysis of the hoof wall can predict values of E moduli consistent with those obtained from theoretical expressions and experimental results. On this basis, FEA represents a valuable modelling technique that can be further employed to developing our understanding

of the structural mechanical relationship at this level of the hoof wall hierarchy.
Given that the FEA modelling technique provides results consistent with those from the modified Hull equation at the macromechanical level, reasonable confidence can be expressed in the theoretical predictions at the micromechanical level. This assertion is further supported by work of Hull (18) and Saliba (19) who recorded stress concentration factors in synthetic composite materials at a volume fraction of .25 and below, similar to those predicted for the hoof wall models at ~2 even in the case of high Ef/Em ratios.

The stress concentration factors predicted within the tubular components are significantly affected by the presence of the medullary cavities. These cavities act as stress concentrators within the structure during loading and their presence results in the generation of stress in the direction of loading. The stiffer the tubular cortex, the lower the stress concentration at these sites due to the reinforcing effect of the cortex. This structural arrangement may serve to protect the medullary cavity from strain and thereby protect the capsule from failure.

The predicted results indicate that at an Ef/Em value > (4 - 4.5) the strain is transferred from the tubule to the matrix, i.e. away from the medullary cavity. At this point the matrix is taking the majority of the deformation; this may represent a means of increasing and preserving the integrity of the structure. The pattern of strain gradient may be instructive in determining the susceptibility to crack production and deviation. However, it is appreciated that perfect bonding at the tubular-intertubular interface has been assumed in these models. If the structural organisation of this boundary were such that a compliant or viscous interface is formed, then, the resultant stress concentration would be significantly reduced.

The presence of a large number of tubules per unit area in the 'outer' region may significantly increase the work of fracture by creating a 'tortuous jagged path' to inward crack propagation Reilly et al (6). As energy is absorbed in the separation of two different phases Vogel (5), the tubular-intertubular interface may produce an effective crack stopping mechanism Bertram and Gosline (21) Reilly et al (8) Kasapi and Gosline (12). Conversely it may be argued that localised stress concentrations jeopardise the material by reducing the total energy required to cause fracture Griffith (22). Thus the tubular density in the 'outer' hoof wall might serve to concentrate stress in this part of the hoof wall, so as to achieve the controlled elimination of damaged hoof wall and protect the capsule from catastrophic failure.

The accuracy of the model is highly dependent upon the assumptions made. The assumptions made in this model were; a hexagonal geometric array, isotropic material properties, linear elasticity, perfect bonding, and ignoring the effects of other levels within the structural hierarchy.

Work is in progress to model this complex material using finite element analysis at the microscopic level of the structural hierarchy, in response to the stress distribution predicted by the gross anatomical model. An accurate model for the hoof wall will enable prediction of changes in the macro and micromechanical performance due to changes in the microstructure, which can occur in certain pathological states and in response to nutrition.

The application of Finite Element Analysis to the structural performance of the hoof wall extends and complements the range of experimental techniques that have formerly been used to evaluate hoof wall function. A better understanding of the function of the hoof as a load-bearing component can be provided by a detailed evaluation of the possible contribution of the various components of the material to its overall performance. In this respect FEA provides the means to assess both the macromechanical and micromechanical performance of the hoof.

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APPENDIX VIII

Equine vet. J., Suppl. **26** (1998) 58-65

Effect of a supplementary dietary evening primrose oil mixture on hoof growth, hoof growth rate and hoof lipid fractions in horses: a controlled and blinded trial

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Keywords: horse; evening primrose oil; maize oil; cod liver oil; hoof; growth; growth rate; lipid

Summary

The lipid chemistry of the normal equine hoof, together with the effect of oral supplementation with an evening primrose oil mixture (EPOM) on its growth, growth rate and lipid content was assessed in a controlled and blinded feeding trial at the Defence Animal Centre. Twelve horses were paired as closely as possible according to sex, age, weight, height and colour and then one from each pair was randomly allocated to treatment or control groups. The treatment group received 30 ml of oral EPOM/day, otherwise the nutrition and management regimes were the same for all horses. No significant differences (P>0.05) were seen between treatment and control groups for hoof horn growth or growth rate. However, there was a significant difference (P<0.05) in hoof horn growth within the treatment group only between weeks 4 and 8 after the start of supplementation. The *stratum medium* contained significantly higher amounts of cholesterol ester (P<0.05), triglycerides (P<0.001) and free fatty acids (P<0.05) than the periople. The periople contained significantly higher levels of free cholesterol and phospholipid (P<0.001) than the *stratum medium* of the hoof wall. There were no significant differences (P>0.05) between treatment and control groups for any of the lipid fractions measured for the *stratum medium* from the clippings of the hoof wall. However, there were differences in perioplic lipid analysis with significant increases (P<0.05) in cholesterol esters and partial glycerides and a significant reduction (P<0.001) in free cholesterol in the treatment group following supplementation.

Introduction

The equine hoof is a highly evolved locomotor organ of epidermal origin (Reilly 1997). The bulk of the hoof wall is modified *stratum corneum*. For skin, the *stratum corneum* is a 2 phase structure consisting of proteinaceous cells which are separated by thin layers of lipids occupying the intercellular space (Chandrasekara and Shaw 1977; Elias 1983). This interstitial lipid phase represents a continuous matrix in which the protein phase, which constitutes by far the larger volume fraction, is discontinuous. The cells provide the physical stability to the horny layer while the intercellular lipids prevent water from penetrating the whole structure (Landmann 1988).

Negishi *et al.* (1977) suggested that lipid should be 'taken into consideration for the protection of the equine hoof' but the importance of lipid to this organ has not been fully elucidated. Reilly *et al.* (1996) suggested that the chemistry of intercellular bonding contributes to the fine function of the hoof capsule. This is because, structurally, lipids are important at surfaces, and in biological membranes, at the barrier between one environment and another (Gurr and Harwood 1996). The physical properties of membranes, strongly influenced by the lipid composition, are regulated by environmental changes such as diet and temperature (Gurr and Harwood 1996). Changes in physical properties may modulate the activities of membrane proteins such as enzymes, transporters of small molecules across the membrane, or receptors for substances such as hormones, antigens or nutrients (Gurr and Harwood 1996). Any of these may affect the regulation of keratinocyte behaviour and ultimately the function of the hoof as a structure.

Grosenbaugh and Hood (1993) suggested that the nature of the lipid matrix within the hoof determines the final material property of the 'finished product'. This can be explained, indirectly, because the stiffening of horn is modulated by water content (Bertram and Gosline 1987; Kitchener 1987) and, directly, because the physical presence of lipid may alter hoof wall modulus according to the volume fraction it occupies. In these ways, the continuous lipid phase is likely to contribute to stress transfer and other forms of communication within the hoof which, together with tubular aspects of hoof morphology (Reilly *et al.* 1996) will be part of a hierarchy of mechanisms which enable the hoof to perform its function.

The periople is a band of soft, tubular horn which passes through a keratohyaline stage in its cornification (Pollitt 1995). It functions to 'cement the skin to the hoof' (Smith 1921) and is thought to prevent undue evaporation from the horn beneath (Smith 1921; Schummer *et al.* 1981). A detailed lipid analysis of the periople has not been reported, although the periople is thought to have a higher lipid content than hoof (Leach 1980; Bolliger 1991).

Grosenbaugh and Hood (1993) and Reilly (1995) have called for more research effort in the fields of hoof horn biochemistry and other processes of keratinisation and cornification because information that relates directly to the hoof is scant. Identifying the lipid types within the hoof capsule would allow their function to

TABLE 1: Lipid fractions Identified in hoof material

Type of lipid identified	Area of/type of hoof	Author
Neutral lipids	Basal and suprabasal cells	Leach (1980), Grosenbaugh and Hood (1993)
Phospholipid	May be present in periople Extracellular matrix	Bolliger (1991) Grosenbaugh and Hood (1993)
Glycolipid	Horse hoof	Grosenbaugh and Hood (1993)
Triglycerides	Cattle hoof Hoof wall/sole/frog Horse hoof	Ueta *et al.* (1971) Negishi *et al.* (1977) Wertz and Downing (1984)
Free fatty acids	Cattle hoof Hoof wall/sole/frog Horse hoof	Ueta *et al.* (1971) Negishi *et al.* (1977) Wertz and Downing (1984)
Cholesterol ester	Cattle hoof Hoof wall/sole/frog Horse hoof	Ueta *et al.* (1971) Negishi *et al.* (1977) Wertz and Downing (1984)
Free cholesterol	Cattle hoof Horse hoof	Ueta *et al.* (1971) Wertz and Downing (1984)
Sterol ester	Horse hoof	Negishi *et al.* (1977)
Sterol	Horse hoof	Negishi *et al.* (1977)
Lactones Hydrocarbons	Horse hoof	Wertz and Downing (1984)
Cholesteryl sulphate	Cattle hoof Horse hoof	Ueta *et al.* (1971) Wertz and Downing (1984)
'Ungulic acid'	Horse hoof	Leikola *et al.* (1969, 1970)

be determined from their association with other measured hoof parameters. Whether they could be manipulated and whether the function of the hoof capsule changes with their manipulation, could then be investigated. However, categorisation of lipid type within the hoof has not been consistent.

Some lipids possess chemical groupings that associate with water (hydrophilic groups), often called polar or amphiphilic lipids in juxtaposition with hydrophobic moieties. In contrast, hydrophobic lipids, without polar groups, are often called neutral, apolar or nonpolar lipids (e.g. triacylglycerols [triglycerides], wax esters and sterols) (Gurr and Harwood 1996). As sterols contain an alcohol function, they may form esters with fatty acids which are among the most hydrophobic of all body lipids (Gurr and Harwood 1996).

That lipid exists within the hoof has been shown by Leach (1980), Bolliger (1991) and Grosenbaugh and Hood (1993) who used simple staining techniques to show the presence of neutral lipid, glycolipid and phospholipids, but they were not quantified. Extraction of lipid material from hoof has been achieved by thin layer chromatography (TLC) and gas liquid chromatography (Leikola *et al.* 1969; Ueta *et al.* 1971 [TLC only]; Negishi *et al.* 1977; Wertz and Downing 1984). Other techniques used to identify lipid in hoof include infra red spectroscopy and mass spectrometry (Leikola *et al.* 1969; Ueta *et al.* 1971; Wertz and Downing 1984). Lipid fractions found in hoof material by other authors are shown in Table 1.

Leikola *et al.* (1969) found the total lipids of the hoof constituted about 2% of the fresh weight, with polar lipids contributing about 50% of the total lipids and 50% made up of neutral lipid. Negishi *et al.* (1977) demonstrated differences in total lipid composition between different areas of the hoof. For the hoof wall, 80% was non polar lipid. Leikola *et al.* (1969) thought that nearly 50% of the lipid in bovine and equine hoof consisted of a ganglioside sulphate, which they termed ungulic acid. However, Ueta *et al.* (1971) questioned the identification of ungulic acid as they found that cholesteryl sulphate was the major lipid in cattle hoof. They concluded that the ungulic acid found by Leikola *et al.* (1969, 1970) was cholesterol sulphate with small amounts of impurities. This was confirmed by Birkby *et al.* (1982) who also found cholesteryl sulphate in cattle hoof.

Free cholesterol and cholesterol esters

In mammals, the lipids involved in membrane structures include unesterified (free) cholesterol, which tends to decrease membrane fluidity (Apps *et al.* 1992). Stubbs and Smith (1990) suggested that the term membrane fluidity has taken on a semi-empirical usage. They recommend that it should only be employed when discussing the rate of motion of a membrane. However, it is also commonly used as a term to describe the order of the components within a membrane. It is used here to describe both the rate of membrane motion and the order of the components which affect it.

Cholesterol is a key regulator of membrane fluidity. It intercalates between the phospholipid components of the membrane, therefore preventing the crystallisation of the fatty acyl chains. High concentrations of cholesterol in membranes sterically block large motions of fatty acyl chains (Stryer 1988). Schmidt *et al.* (1991) found that inhibition of cholesterol synthesis abolished not only increases in cellular cholesterol but also spontaneous cholesterol ester formation, indicating there might be a direct link between cholesterol and cholesterol ester formation.

Cholesterol esters are more hydrophobic than, and behave differently to, free cholesterol. They are absent from membranes but function as storage forms of cholesterol (Apps *et al.* 1992). In skin, a minor portion of free cholesterol is sulphated by the enzyme cholesterol sulphotransferase to form cholesterol sulphate (Epstein *et al.* 1984, cited in Schurer and Elias 1991). Wertz and Downing (1984) concluded that cholesteryl sulphate is the major polar lipid of horse hoof and suggested that its presence in such high proportion in a fully keratinised tissue may be of functional significance. It has been suggested that cholesteryl sulphate may cement together horny cells (Epstein *et al.* 1981; Elias 1983; Williams 1983) and may be important, therefore, in determining the cohesive properties of epidermal keratinocytes (Shapiro *et al.* 1978; Smith *et al.* 1982; Williams 1983) and ultimately, therefore, the cohesiveness of the hoof as a structure. Ueta *et al.* (1971) also commented that relatively large quantities of cholesterol sulphate in keratinous tissues suggests that these tissues play a role in the excretion of cholesterol as a form of sulphate ester but, more recently, Williams (1991) has suggested that cholesterol sulphate may be a regulator for cholesterol synthesis where lipids are generated for a barrier function.

Triglycerides

Triglycerides are the simplest form of natural lipids and are also

TABLE 2: Details of animals used in the trial

	Treatment group						Control group					
Pair	Name	Sex	Age (yr & mo)	Weight (kg)	Height (hh)	Colour	Name	Sex	Age (yr & mo)	Weight (kg)	Height (hh)	Colour
1	Quantum Leap[1]	M	5	669	16.1	Brown	Prestige[1]	G	5.9	597	16.3	Brown
2	Quasar	G	5.8	583	16.2	Chestnut	Squire Cheatham	G	5.2	656	17.1	Brown
3	Qualifier	M	6.2	628	16.3	Brown	Pirouette	G	7.1	561	16.2	Chestnut
4	Quantock	G	4.9	574	16.3	Brown	Quatermass	G	5	560	16.3	Bay
5	Quizmaster	G	4.9	675	16.3	Light Bay	Calypso	M	4.9	562	16.3	Chestnut
6	Quality Street	G	5	582	16.1	Black	Quadraphenia	G	5.1	581	16.1	Dark Bay

M: mare; G: gelding. yr: years; mo: months; [1]Bedded on shavings and fed haylage instead of hay.

termed triacylglycerols (Apps *et al.* 1992). Glycerol has 3 hydroxyl groups, each of which combines with 3 fatty acids to form a triacylglycerol by a condensation reaction. The link is supplied by an ester bond (Carr and Cordell 1992).

Phospholipid

A phospholipid is formed when one of the 3 fatty acids on the glycerol backbone is exchanged for a phosphate. The phosphate group is hydrophilic but is still attached to hydrophobic lipid molecules, therefore one part seeks to interact with water and the other part avoids water. In a lipid bilayer membrane, the hydrophilic phosphate 'heads' orientate themselves on the outside with the hydrophobic fatty acid 'tails' on the inside, forming 2 layers, with the fatty acid tails preventing water molecules from passing across the bilayer (Carr and Cordell 1992).

Grosenbaugh and Hood (1993) believed that phospholipids comprise the majority of the cornified extracellular matrix of the hoof wall. Since no data were given in support, this belief was presumably based on staining with acid haematin which gave a different staining pattern in the hoof wall compared to human *stratum corneum*, where the extracellular matrix from the soft and flexible epithelium is composed of neutral lipids (Elias 1983). Phospholipids may be present within the membrane coating granules of hoof (Leach 1980) whose discharged contents act as 'intercellular cement' (Matoltsy and Parakkal 1965). No chemical bonds link phospholipids to each other and therefore each molecule is free to move independently, providing fluidity and, therefore, flexibility to membranes (Vander *et al.* 1990).

Free fatty acids

Fatty acids exist as subunits of triacylglycerols, phospholipids or as 'free' molecules (Vander *et al.* 1990). Fatty acids are composed of a long chain of carbon atoms combined with hydrogen, with a carboxyl group at the end of the chain. They may have a number of carbon atoms (Carr and Cordell 1992).

Some fatty acids are termed 'essential'. These are polyunsaturated and cannot be synthesised in the body and must, therefore, be provided in the diet (Stubbs and Smith 1990). Rich sources of essential fatty acids (EFAs) are evening primrose oil (EPO), maize oil (MO) and cod liver oil (CLO). EPO provides γ-linolenic acid, MO provides Omega-6 linoleic acid and CLO provides Omega-3 fatty acids such as α-linolenic acid. The metabolites of EFAs have important functions in prostaglandin and leukotriene production (Horrobin 1992) but EFAs also have an important structural role to play in the intermembraneous environment. They are essential constituents for all cell membranes and confer on them the properties of fluidity, flexibility and selective permeability (Horrobin 1992). It is also known that the lipid composition of cell membranes is highly susceptible to dietary manipulation (Stubbs and Smith 1990).

Effects of orally supplemented oils on keratinising epithelia

A lack of, or abnormal metabolism of, EFAs may influence every cell and therefore every system in the body as changes in membrane lipids can also affect the structure and behaviour of proteins in the membrane (Mitsuhashi *et al.* 1986). EFAs control the water permeability of the skin, regulate cholesterol and transport synthesis, and are also required for cell division (Horrobin 1992).

Although insufficient EFAs in the diet have been associated with poor skin and hair health in other species, the EFA requirements for horses are not yet known (Anon 1989). Elias (1981) stated that EPO corrected the symptoms of pathologically dry skin and Campbell and MacEwan (1982) suggested that treatment with oral EPO caused a reduction in symptoms of brittle and splitting nails in humans within one or 2 months.

In view of the importance of lipid in other epithelia and since epidermal intercellular lipids are susceptible to dietary manipulation (Stubbs and Smith 1990), a trial was conducted with the null hypothesis that supplementation with an EPO mixture (EPOM) would not alter the growth characteristics or lipid composition of hoof horn or periople in the horse. The aims of the study were to implement a match-paired and blinded feeding trial to produce hoof horn under controlled conditions so that information on the normal lipid content of hoof *stratum medium* and periople could be gained, and then to assess the effect of EPOM supplementation on growth, growth rate and lipid composition of the hoof.

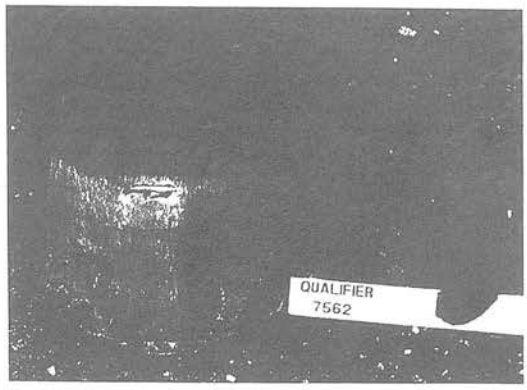

Fig 1: Dorsal view of hoof to show perioplic sampling site.

Materials and methods

Experimental design

Twelve Irish draft cross Thoroughbred hunter type Army horses were brought in from the field having had 6 months at grass. They had their feet trimmed for mediolateral balance and were then paired as closely as possible according to sex, age, weight, height and colour (Table 2). The horses were on an increasing plane of nutrition for the first 2 weeks of the trial (i.e. the basal period). At the end of this period each horse received 15 lbs of hay or haylage split into 2 feeds daily, and 15 lbs of concentrate feed split into 3 feeds daily. The concentrate consisted of Fibre P[1], Horse and Pony Nuts[2], and Cavalry Mix[1], fed in a ratio of 2:3:1 respectively by weight. The oil contents in the dry matter of each feed were 2, 3 and 2.5%, respectively. In each case, this was provided by soya bean oil.

One of each pair was randomly assigned to the treatment group to receive 30 ml oral EPOM supplement daily. This was mixed into its evening concentrate ration. Each 30 ml EPOM fed had the components given in Table 3. This was based on a small animal product that was manufactured by the sponsoring company. However, since the aim was to investigate the effect of supplementation with oils only, the normal vitamin component of the multivalent product was removed. The dose rate fed to the horses was based on the small animal dose extrapolated for bodyweight. The horses not supplemented were the control group. Only one author (JDR), who had the responsibility for the management of the trial, knew which animals were in the control and treatment groups. Collection of data was carried out without recourse to this information.

Supplementation began on the 30th October and continued for a period of 164 days. No problems were experienced with acceptance of the EPOM supplement. The management regimes, exercise surface, intensity and duration of work, were virtually identical for all the horses in the trial. They were individually stabled and bedded on straw with the exception of 2 horses, which were bedded on shavings to alleviate respiratory problems (Table 2). Hoof dressings were not applied during the trial period. Due to work commitments, one horse from the treatment group left the trial in the third period and one control horse left the trial in the fourth period. Hoof growth measurements were taken at periodic intervals.

TABLE 3: Components of the evening primrose oil mixture (EPOM)

Component	Volume fed daily (ml)
Evening primrose oil	18.75
Maize oil	8.15
Cod liver oil	3.00
Tween 80†	0.09
BHT††	0.01
Total	30.00

†Tween 80, (sugar + oil + fatty acid), helps with forming an emulsion and is retained to aid absorption. It also has surfactant properties and ensures the product remains clear.

††BHT is a standard anti-oxidant (butylated hydroxy toluene). It particularly helps stabilise the γ-linolenic acid and is retained because the latter is fairly labile.

Periodic hoof growth

The growth of horn at the midline dead centre (MDC) of the left fore foot of all horses in the trial was measured according to Reilly *et al.* (1996, 1998). The hooves were initially brand marked 40 mm from the distal hair line. When the hoof wall had grown enough to cause the marks to be near the horseshoe, the hooves were branded again.

Sampling of stratum medium *and periople for lipid analysis*

Hoof clippings and perioplic samples were taken 12 weeks after the start of supplementation (10 horses; 5 from the control group, 5 from the treatment group). Hoof clippings were removed according to Cope *et al.* (1998) and a sample of the *stratum medium* was used from the MDC of the clipping. Full thickness periople samples were removed at the proximal MDC using a scalpel (Fig 1). Samples were analysed for cholesterol ester (CE), triglycerides (TG), free fatty acids (FFA), phospholipids (PL), free cholesterol (FC) and partial glycerides (PG) by high performance thin layer chromatography and gas chromatography. Results were expressed as percentages of total lipid extracted. Samples from the control group only were used to compare lipids within the *stratum medium* and the periople.

Statistical analysis

All data were analysed using Minitab[3]. The data set was checked for normality using normal probability plots. Normal data were analysed using *t* tests and ANOVA; and non-normal data were analysed using Mann-Whitney U tests. Significant differences were assessed at $P<0.05$.

Results

Hoof growth and growth rate

All data for hoof growth were normally distributed ($P>0.05$). There was no significant difference ($P>0.05$) between the treatment and control groups for growth at the end of the basal period (Figs 2, 3) and there were no significant differences ($P>0.05$) in mean cumulative hoof growth (Fig 2) nor in mean periodic hoof growth (Fig 3) between treatment and control groups for any of the sampling periods. However, for the treatment group

TABLE 4: Quantified lipid fractions found in hoof material

	Results from this work				Other authors					
	Control	Treatment	Control	Treatment	Leikola *et al.* (1969)	Wertz and Downing (1984)		Negishi *et al.* (1977)††		Ueta *et al.* (1971)
	Horse hoof clipping	Horse hoof clipping	Periople	Periople	Horse hoof	Horse hoof clipping	Hypony-chium	Horse hoof wall	Sole	Frog	Cattle hoof
Lipids as % FW					2.00			0.58	0.86	0.97	
Lipids as % DW						1.50	1.90				
Cholesterol ester	17.28†	17.88†	9.35†	12.03†		1.60	4.80				
Triglycerides	23.73	26.10	7.64	7.96		3.20	0.10	26.60	25.30	22.20	14.00
Free Fatty acids	29.76†	27.99†	7.07†	9.33†		15.60	3.10	12.80	12.50	8.40	24.00
Free cholesterol	7.29	9.79	19.19	12.96		36.60	39.80				31.00
Phospholipids	24.36	29.32	48.51	46.48							Negligible
Partial Glycerides	0.00	0.00	7.29†	10.85†							
Total ceramides						10.00	13.70				7.00
Squalene						1.40	11.70				
Sterol ester								32.80			
Sterol								15.40			
Diglyceride											3.00
Glucocerebroside											6.00
Lactones						0.40	0.40				
Hydrocarbons						8.80	0.20				
Cholesteryl sulphate						19.60	15.30				10.00
Ungulic acid					Identified						
Polar lipids					50.00			20.00	19.00	18.00	
Nonpolar lipids					50.00			80.00	81.00	82.00	
Unknown nonpolar								12.10	17.65	14.50	
Unidentified						23.00	9.00				4.0

Results are expressed as means except†, where they are expressed as medians. Results are expressed as percentages of total lipid except††, where they are expressed as a percentage of nonpolar lipid. FW: fresh weight; DW: dry weight.

only, there was a significant difference for periodic mean hoof growth between periods 2 and 3 ($P<0.05$) (Fig 3).

The growth results from this work, converted to give a growth rate in mm/day/period of trial, are shown in Table 5. There was no significant difference between periodic growth rates at any time during the trial ($P>0.05$). The mean growth rates over the whole period of the trial for the treatment and control groups were 0.18 (± 0.09) mm/day and 0.21 (± 0.07) mm/day respectively.

Lipid analysis

Results for quantified lipid fractions found in hoof material in this study, together with those of other authors, are given in Table 4. The results are shown as percentages of total lipid extracted. The data sets for TG, FC and PL were all normally distributed but the data for CE, FFA and PGs were not.

TABLE 5: Mean hoof horn growth rate during the trial periods

Period of trial	Treatment (mm/day)	Control (mm/day)
1	0.31	0.34
2	0.12	0.18
3	0.26	0.16
4	0.12	0.21
5	0.11	0.18
Means of means	0.18	0.21
Standard deviation	0.09	0.07
Coefficient of variation	0.51	0.34

Lipid content of stratum medium *and periople*

Comparison of the lipid fractions in the *stratum medium* and periople for the control group only is shown in Figure 4. The *stratum medium* contained significantly higher amounts of CE ($P<0.05$ by Mann-Whitney *U* test), TG ($P<0.001$ by *t* test) and FFA ($P<0.05$ by Mann-Whitney U test) than the periople. The periople contained significantly higher amounts of FC and PL ($P<0.001$ by *t* test) than the *stratum medium*.

The periople was found to contain partial glyceride but none was registered for the *stratum medium* (Table 4, Figs 4, 5 and 6). The lipid make up for the *stratum medium* for control animals in descending order of proportion of total lipid extracted was: FFA, PL, TG, CE and FC. For periople it was: PL, FC, CE, TG, PG and FFA.

Treatment effects

The *stratum medium* showed no significant differences between treatment and control groups for analysis of TG, FC and PL ($P>0.05$, *t* test) and CE and FFA ($P>0.05$, Mann-Whitney *U* test)

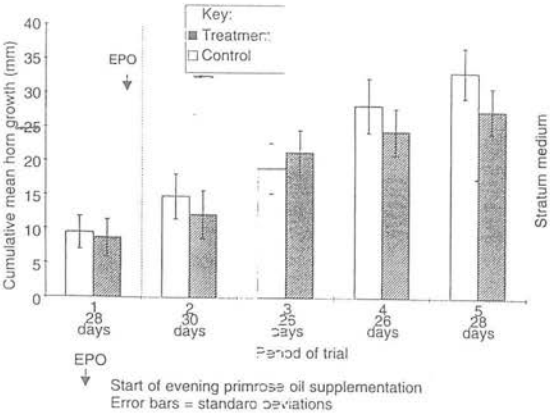

Fig 2: Cumulative mean hoof horn growth for left fore feet for treatment and control groups.

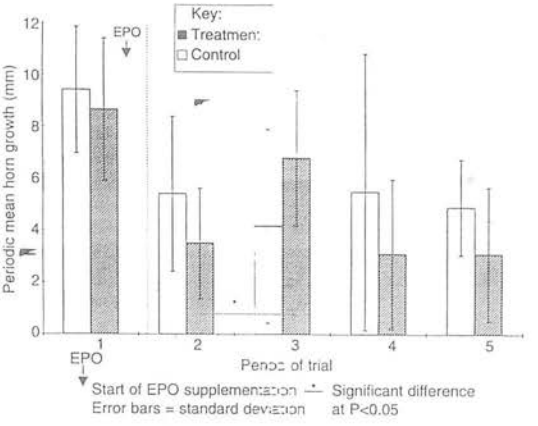

Fig 3: Periodic mean hoof horn growth for left fore feet for treatment and control groups.

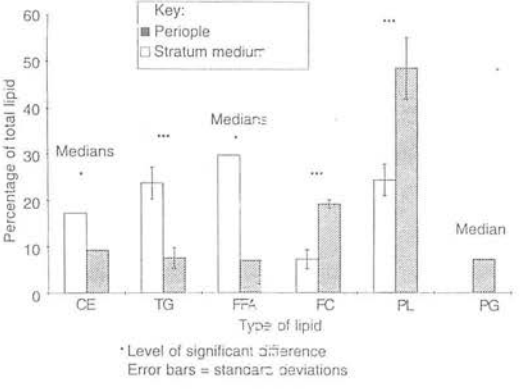

Fig 4: Comparison of stratum medium *and periople mean and median lipid content as a percentage of total lipid identified for control animals only.*

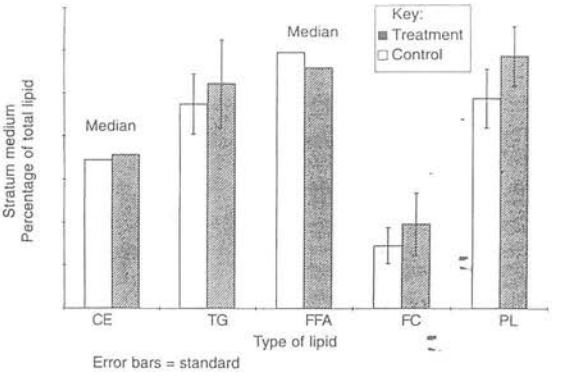

Fig 5: Comparison of stratum medium *mean and median lipid content for treatment and control animals as a percentage of total lipid identified.*

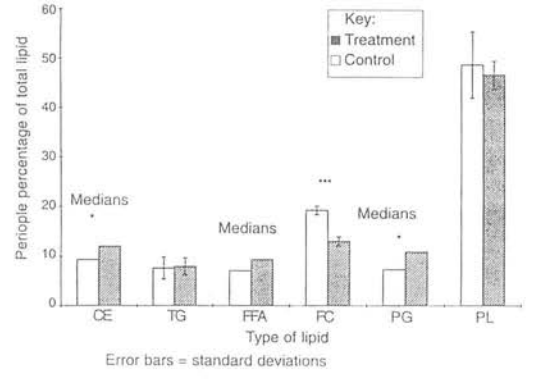

Fig 6: Lipid analysis of periople for treatment and control animals.

(Fig 5). Supplemental EPOM did not, therefore, affect the lipid composition of the *stratum medium* of equine hoof.

There was a significant increase in perioplic CE ($P<0.05$ by Mann-Whitney *U* test) and PG ($P<0.05$, Mann-Whitney U test) for the treatment group compared to the control group and there was a significant decrease in the level of perioplic FC ($P<0.001$ by *t* test) (Fig 6).

Therefore, supplemental EPOM affected the lipid composition of the periople of equine hoof in this trial.

Discussion

Hoof growth and growth rate

There was no significant difference for mean hoof growth between treatment and control groups during the basal period of the trial. However, hoof growth was higher at this time than for any of the remaining trial periods. A possible explanation for this would be that trimming in the basal period may have stimulated hoof growth as has been shown in cattle (Vermunt and Greenough 1995). Another contributing factor may have been the increasing plane of nutrition during this period.

There was no significant difference between treatment and control groups for cumulative mean hoof growth, but there was

a trend for EPOM supplementation to produce lower cumulative mean horn growth for all periods of the trial except in period 3 when this trend was reversed (Fig 2). The explanation for this is found when periodic mean horn growth is considered (Fig 3). Here, a significant surge in growth (a rise of nearly 100% of the amounts grown in other periods of the trial) is seen for the treatment animals only. This is significantly different to growth in period 2 ($P<0.05$). The difference in mean ± s.d. growth in period 3 of 6.8 mm ± 2.61, compared to 3.5 mm ± 2.12, and 3.1 ± 2.8, in periods 2 and 4 may be of practical use where a surge in growth is required, followed by a return to relatively lower growth compared to a control population.

The control group mean hoof growth rate of 0.21 mm/day (Table 5) is within the ranges quoted by Glade and Salzmann (1985), and Geyer and Schulze (1994). For growth rate, EPOM supplementation in this work has given a response, within treatment animals only, of 0.26 mm/day compared to a mean growth rate of only 0.12 mm/day in the period before, in hunter type horses. With biotin supplementation the treatment effect was to produce a mean growth rate, over 5 months, of 0.19 mm/day, compared to 0.16 mm/day for nonsupplemented animals, in this case with ponies (Reilly *et al.* 1998).

Such changes, however, are subtle and the full extent of the capsular response to supplementation may take some time to understand or appreciate fully, and the effects (if any) on the material and histological features of the capsule need to be known before recommendations for supplementation can be made.

The timing of the response seen in the treatment animals only in this study agrees with the findings of Campbell and MacEwan (1982), who supplemented human patients with EPO, and it is different to the timing of another growth effect shown in horse hoof with biotin by Buffa *et al.* (1992) and Reilly *et al.* (1998). This may reflect differences brought about in the metabolic processes of keratinisation which, for the hoof, are far from fully understood.

What these results do show however, together with the results of Buffa *et al.* (1992) and Reilly *et al.* (1998), is that the hoof capsule is capable of different responses when the horse is supplemented with different nutrients. Therefore, evidence is accumulating from controlled work, for a nutrient-hoof horn axis in terms of growth and growth rate responses.

Lipid chemistry

In this work, the results are expressed as proportions of total lipid extracted. Negishi *et al.* (1977) presented their data as a percentage of neutral lipid. If there had been an increase in total lipid this would not be shown and absolute measurement of lipid content is suggested for future work. Wertz and Downing (1984) used whole hoof clippings. Our results are restricted to the MDC portion of the clipping. It is unclear which part of the wall Negishi *et al.* (1977) used, or whether *stratum medium* and periople were separated.

Comparative lipid content of stratum medium *and periople*

Phospholipids have not been previously quantified in equine hoof. The results show that phospholipids do not comprise the majority of the extracellular matrix of the hoof wall as thought by Grosenbaugh and Hood (1992). For the control group, phospholipid comprised 24.36% of the total lipid content and FFA content was higher at 29.76%.

However, our results show that the periople does contain more phospholipid than the *stratum medium* (48.51% phospholipids in periople vs. 24.36% in *stratum medium* - see Table 4).

Since the periople passes through a keratohyaline stage in its cornification (Pollitt 1995) and keratohyaline granules are a complex of calcium, phospholid and protein (Spearman 1973), this may account for the high phospholid content in the periople. Also the process of cornification is accompanied by a marked decrease in phospholipid content (Wertz and Downing 1991), which explains the significantly lower ($P<0.001$) amount of phospholipid in the fully keratinised *stratum medium* than in the less highly keratinised periople.

As phospholipid acts to prevent water molecules from passing across a lipid bilayer membrane (Carr and Cordell 1992), the assumed role of the periople in acting as a barrier to maintain hoof moisture content (Smith 1921; Schummer *et al.* 1981) may be justified on chemical grounds.

Treatment effects on stratum medium *and periople*

There was no effect of EPOM supplementation on any lipid fractions measured in the *stratum medium* of the MDC of the clipping. However, a sampling time of 12 weeks would not have allowed new horn to grow to the sampling site and an extended period of trial, to allow full capsular renewal, is recommended, therefore, for future work. Butler (1976) believed that transfer of metabolites occurs within the hoof capsule and, in other nutritional trials, changes have taken place at the distal border of the hoof prior to new growth reaching the ground (Comben *et al.* 1984; Reilly and Brooks 1990; Buffa *et al.* 1992). This was not the case for the lipid measured within the *stratum medium* of clippings in this trial.

However, the periople from EPOM supplemented animals has shown significant differences in lipid content compared to controls. This is the first report of supplementation giving rise to a change in hoof lipid and provides direct evidence for a nutrient-hoof horn axis. The significance of an increase in CE and PG and a decrease in FC is not fully understood. However, the existence of a biochemical route that is available for nutritional manipulation may have profound effects for our understanding of hoof function. Changes in important hoof constituents, such as CE and FC, may have given rise to changes in membrane fluidity. Although membrane fluidity was not measured in this work, theoretically an increase in CE and a decrease in FC acts in concert to increase fluidity and therefore alter receptiveness to biochemical regulators. This may account for the change in growth in the treatment group and may have other structural or material effects. The significance of this to hoof physiology and function requires further work. Further trials are also required in order to elucidate whether or not specific components of the orally supplemented EPOM had more influence in bringing about the particular results found in this work. In view of the accumulated evidence, from controlled work, for the existence of a nutrient hoof horn axis, further nutritional trials are now justified as the results from this work could have important implications for hoof management and animal welfare.

Acknowledgements

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Manufacturers' addresses

[1]Fibre P and Cavalry Mix - Dodson and Horrell Ltd, Ringstead, UK.
[2]Spillers Horse Feeds, Dalgety Agriculture Ltd, Milton Keynes, UK.
[3]Minitab version 11.11, Minitab Inc, PA, USA.

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